On-Chip Food Safety Monitoring:

Multi-analyte Screening with Imaging Surface Plasmon Resonance-based Biosensor

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Sabina Rebe Raz

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To my parents,
Dr. Yefim and Dr. Alla Rebe.
With utmost love, respect and appreciation.

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Chapter 1

Introduction to This Thesis

The main goal of the study described here was to develop an imaging Surface Plasmon Resonance–based biosensor, for multiplexed and quantitative detection of different health-threatening compounds in food. Here the motivation to conduct this study is briefly introduced, together with the scope and the outline of this thesis.

Motivation

To protect the health of consumers, food safety is routinely monitored. A great deal of research is focused on the development of adequate analytical methods, which will enable identification of numerous food components: e.g. allergens, residues of veterinary drugs, pesticides and environmental contaminants, vitamins, additives and minerals in order to determine whether the food product meets statutory norms or company standards. Such an extensive screening procedure, however, is a labour-intensive, time- and (bio) chemical consuming task which is generally hampered by the limited number of compounds that can be simultaneously measured with the conventional analytical techniques. Additionally, since food hazards include many fundamentally different agents, e.g. microorganisms, proteins and small molecules, the conventional techniques used for their detection require specialized laboratories and personnel. For example, pathogens are detected by culturing techniques followed by biochemical and serological identification, whereas antibiotic residues are detected using high pressure liquid chromatography (HPLC) and mass spectrometry (MS) techniques. A good alternative is a more generic detection platform which is based on a biorecognition element (bioassay). Antibodies are the most commonly applied biological recognition elements in bioassays today, due to their specificity, affinity, stability and availability. Antibodies can be directed against different antigens, e.g. epitopes from bacterial cell wall, allergens and antibiotics, offering powerful tools for the detection of wide variety of health-threatening agents in food. In classical immunoassays, the binding event is detected via various labels (fluorescent dyes, enzymes or radioisotopes) attached to antigens or antibodies. The immunoassay set-up currently dominating the routine laboratory analysis is the enzyme-linked immunosorbent assay (ELISA) in the 96 well plate format. Pioneered in 1971, this almost forty years old invention still has an enormous impact in the bioanalytical field. During the last decade, a certain degree of automation and a higher throughput was achieved, via implementation of bar-code labelling and automatic washing, and pipetting systems. However, ELISA still remains a time and reagents consuming, and labour intensive screening technique. The bottleneck of the limited amount of analytical data that can be acquired in a single measurement together with the costs and time-consumption of the analysis, could be alleviated when a high throughput screening technology would be available. Recent developments of surface plasmon resonance (SPR) technology in the direction of high-throughput systems presents a promising alternative to the classical immunoassay formats. One of such systems is imaging SPR (iSPR). Spatial modification of the surface, by microarraying, in

combination with iSPR allows label free and multiplexed analysis in a single measurement. Utilisation of the iSPR technology as multi-analyte device in a new screenchip platform, will enable quantitative, rapid and automated food analysis. When applied, it will provide the end-user with a detailed food product profile, contributing to the decision making process on the quality and safety of foods.

Aims and scope

Analytical systems today show a varying degree of miniaturisation and significant progress has been made in research and development of lab-on-a-chip "building blocks". To reach a fully integrated lab-on-a-chip analytical device, certain elements of a system are usually miniaturised in an individual manner. This thesis dealt with the miniaturization of the bio-assay element to a form of a multi-analyte screening chip. Whereas high throughput screening technology has become readily available for genomics and proteomics applications, high throughput determination of contaminants in foods still awaits solutions. The main goal of this study was to develop an iSPR–based biosensor, for multiplexed and quantitative detection of different health-threatening compounds in food. This chip-based system would enable label-free, real-time, automated and simultaneous detection of multiple target analytes within minutes. The major scientific challenge of this work was the combination of the microarray and SPR technology for an analytical application, with main subtasks as follows: 1- Adaptation of the IBIS iSPR platform for automated and robust measurements. 2- Miniaturization of the immunoassay on the iSPR sensor chip, mainly improving ligand immobilization conditions in a microarray format. 3- Multiplexing immunoassays on the micro-scale. 4- Application of the biosensor to "real" samples analysis, including minimizing the interference of food matrix components.

Thesis outline

- **Chapter 1** *"Introduction to this thesis"* provides a short description of the motivation to conduct study, it's aims and the scope and the outline of this thesis.
- **Chapter 2** *"Multiplexed Bioassay-based Approaches to Food and Environmental Contaminants Analysis"* provides a comprehensive literature review of currently available multiplexed bio-assays applied for detection of various food and environmental contaminants. Different multiplex technological platforms are described and compared. Examples of the applications of these technologies for detection of different hazardous agents in food are given as well.

- **Chapter 3** *"Imaging Surface Plasmon Resonance-based Biosensing"* gives a scientific background on Surface Plasmon Resonance (SPR) phenomenon and its utilization in biosensors with a special emphasis on the aspects important for iSPR-based bioassay development.
- **Chapter 4** *"Development of a Biosensor Microarray Towards Food Screening Using Imaging Surface Plasmon Resonance"* deals with the possibilities of implementing iSPR sensor for food analysis. Intrinsic optical properties of the sensor together with direct and competitive immunoassay formats were evaluated in order to demonstrate proof of principle of quantitative screening of high and low molecular weight analytes.
- **Chapter 5** *"Label-free and Multiplex Detection of Antibiotic Residues in Milk Using Imaging Surface Plasmon Resonance-Based Immunosensor"* describes the development of a microarray biosensor, based on iSPR, for quantitative and simultaneous immunodetection of different antibiotic residues in milk. Model compounds from four major antibiotic families: aminoglycosides (neomycin, gentamicin, kanamycin and streptomycin), sulfonamides (sulfamethazine), fenicols (chloramphenicol) and fluoroquinolones (enrofloxacin) were quantitively detected using a multiplexed competitive immunoassay on single sensor chip.
- **Chapter 6** *"Food Allergens Profiling with Imaging Surface Plasmon Resonance-based Biosensor"* describes the development of a microarray biosensor, based on iSPR for rapid, quantitative, and multi-analyte food allergens detection. An allergen profile, comprised of 12 major allergens, of cookies and dark chocolates, was obtained via a direct immunoassay on a single sensor chip within several minutes and with a sensitivity comparable to ELISA.
- **Chapter 7** "*Nanopatterned Submicron Pores On a Gel-supported Membrane For On-chip Sample Preparation in Surface Plasmon Resonance Sensing"* describes a novel approach to tackle the most common drawback of using SPR for analyte screening in complex biological matrices - the nonspecific binding to the sensor chip surface. By depositing a perforated membrane on a polymeric gel structure that exceeds the evanescent wave penetration depth, a filter was created above the sensing region that prevents the diffusion of large particles or aggregates that bind non specifically to the polymeric gel and interfere with SPR sensing, thus

increasing assay's sensitivity and robustness, reducing sample preparation steps and shortening the analysis time in total.

- **Chapter 8** *"Conclusions and Future Perspectives"* offers concluding remarks on the application of iSPR to food screening, suggestions for future research in the field of biosensors and their application prospects for food bioanalytics.

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Chapter 2

Multiplexed Bioassay-based Approaches to Food and Environmental Contaminants Analysis

To guard public health, the food chain and the environment are continuously monitored for the presence of various health threatening compounds. Bioassays provide powerful tools for the rapid screening of large numbers of samples when traditional analytical methods are too cumbersome. Due to their simplicity and/or high-throughput capacity, bioassays are applicable both for screening at critical control points in the field and in monitoring laboratories. Recent advances in miniaturization of analytical systems as well as newly emerging technologies offer alternative platforms with higher automation and multiplexing capabilities for traditional bioassays. Multiplexed bioassays provide control agencies and food industries with new possibilities for improved and more efficient monitoring of food and environmental contaminants. This chapter deals with planar and suspension array technologies and their applications in food and environmental contaminant analysis, focusing on the detection of pathogens, food allergens and proteinaceous adulterants, toxins, antibiotic residues and environmental contaminants.

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1 Introduction

The presence of contaminants in our food and environment became a fact of life. Accidental or deliberate introduction of potentially harmful substances into the agricultural and food chains adversely affects animals, plants and us [1]. Public health concerns, attributed to food and environmental contamination, include increased cancer risk, endocrine, reproductive and neurobehavioral systems disruption, teratogenesis, antibiotic resistance and even death in cases of acute poisoning or anaphylactic shock [2-4]. Some of the contaminants (e.g. pathogenic microorganisms and toxins) can even be used for biological warfare, and thus their monitoring is important for biosecurity as well. Besides health issues, food safety and quality have an economical impact on the food industry, were quality control expenses amount to about 1.5 – 2 % of the total sales [5]. The standard approach towards environmental contamination and food safety management is to legislate and regulate after identifying and quantifying the potential risk [6]. For successful monitoring of levels and trends of contaminants in our environment and food and determination of their significance with regard to public health powerful analytical methods are applied. These include traditional analytical methods, such as gas and liquid chromatography coupled with mass spectrometry, classical microbiological culturing methods coupled with biochemical and serological identification, electrophoresis and immunoassays in traditional formats (e.g. immunoblots and radiolabeled immunoassays) [7-9]. Since more and more products nowadays contain multiple and processed ingredients, which are often shipped from different parts of the world, and share common production lines and storage spaces, food safety and environmental monitoring becomes a challenging task. Currently, it is a common practice to first screen a large number of samples for a possible contamination and then subject the positives to further confirmation. Traditional analytical methods require dedicated laboratories, equipment and highly trained personnel for detection and identification of each type of hazardous agent (e.g. antibiotics, bacteria, allergens). Therefore, prevailing screening tools today are based on assays incorporating biological recognition elements (bioassays), offering a more simplified and rapid analysis [10-14]. Most commonly used bioassays for routine monitoring are enzyme linked immunosorbent assays (ELISAs) in a 96 well plate format. Even though some level of automation has been achieved in the recent years, ELISAs remain laborious, time-consuming and expensive, when multiple targets need to be screened for. Thus, there is a growing need for new multi-analyte screening methods, which will enable rapid and simultaneous detection of contaminants in numerous samples. This review provides an

outlook on the recent developments in bioassay-based multiplexed technologies and their applications for food safety and environmental monitoring.

2 Multiplex Technologies

2.1 Planar Array Technologies

The interest in planar array technologies for food and environmental analysis, with fluorescent, bioluminescent or chemiluminescent (CL) labels for detection, as well as the direct (label-free) detection, is increasing. The microarrays and/or multi-channel platforms offer high multiplexing capabilities for the analytical bioassays, which are particularly useful when multi-analyte screening is needed. Short measurement times, automation, reduced sample volumes and high sensitivity are among the main advantages offered by such systems. The most prominent planar array technologies that have been already applied to food and environmental analysis include the Naval Research Laboratory (NRL) array biosensor based on total internal reflection fluorescence (TIRF) [15], the CL microarray [16] and the Surface Plasmon Resonance (SPR)-based biosensor [17].

2.1.1 NRL Array Biosensor

The NRL array biosensor is based on a planar waveguide that directs evanescent light excitation to fluorophores which are bound to the surface [15, 18, 19]. It is composed of three parts: an array of immobilized molecular recognition elements (usually antibodies), an image capturing and processing system and a fluidics handling unit (Figure 2.1a). Array sensor optics comprise a patterned glass slide, which is placed on a support and is illuminated by launching 635 nm light from a diode laser into one end, a GRIN lens array, which focus the fluorescent patterns, an emission filter, which rejects unwanted laser light and a Peltier-cooled CCD camera which images the array. Due to limited penetration depth of the evanescent wave, only fluoresce of the fluorescent probes which are close to the waveguide surface is measured. This approach, in combination with fluorescent dyes, which are excited at longer wavelengths (e.g. Cy5 and AlexaFluor 647), significantly reduces the interference from the bulk fluid, offering better reproducibility and sensitivity when used for analysis in complex sample matrixes. Optical properties of such fluorophores also allow a small and lightweight source of excitation (e.g. diode laser) and a compact optical detection system (e.g. CCD camera). Multi-analyte detection is achieved through the “bar-code” approach. Biotinylated capture antibodies are immobilized on the avidin-coated waveguide surface in columns, using a Polydimethylsiloxane (PDMS) block with several channels. Then the PDMS block is

oriented perpendicularly and several different samples are passed through the channels. Each sample encounters the "bar-code" of columns, where binding of the analytes to their specific antibodies takes place. The detection is done in real-time, by incubation with reporting fluorescently–labelled antibodies, producing fluorescence in squares were the immuno-complexes are formed [20]. Both sandwich and competitive immunoassay formats have been applied in this system, for the detection of both high and low molecular weight compounds [21]. Currently, a portable and automated version of the array biosensor is available [22] with a detailed protocol for the development of fluoroimmunoassays [23]. The array biosensor has been used for the detection of small molecules [21], toxins [24], proteins [19], bacteria [25] and viruses [26] serving numerous applications in food safety, diagnostics, homeland security and environmental monitoring.

2.1.2 CL- Arrays

The parallel affinity sensor array (PASA) has been developed by Weller *et al.* for multiplexed detection of environmental contaminants in water [16] and antibiotics in milk [27]. The PASA is based on a CL read-out of the microarray via the CCD camera and includes a flow cell, and an integrated fluidic system for reagents handling (Figure 2.1b). The disposable chips, microarrayed with haptens or antibodies, are inserted to the flow cell and places in the dark chamber. The lens and cooled CCD detector are situated beneath the flow cell. Two syringe pumps manage the liquid handling, allowing a nearly permanent flow by an alternating action. Conventional microscope glass slides, modified with (3-glycidyl-oxypropyl)trimethoxysilane, are used as a solid phase support for the microarray. The haptens are microarrayed using a conventional non-contact spotting technique based on piezoelectric nanopumps, producing spots in the diameter of ~350 μm. Multi-analyte immunoassays in an indirect competitive ELISA format can be implemented for rapid and automated multiplexed analysis. Usually, the sample is premixed with a specific antibody and injected over the hapten – microarray. Free antibody binds to hapten spots and then the secondary antibody, labeled with peroxidase (POD), is introduced. Formation of the immunocomplex is detected by light emission in the presence of a luminol-based POD substrate. The direct immunoassay format was demonstrated in this system as well [16]. The microarrays are of multiple use, and are regenerated between sequential measurement cycles. The analyzed samples do not require enrichment and pretreatment steps and the target analytes can be detected within 5 minutes (excluding the regeneration cycle) with sensitivities reaching ng L^{-1} analyte concentrations. A new fully automated and stand-alone version of this system has been recently reported for field applications [28].

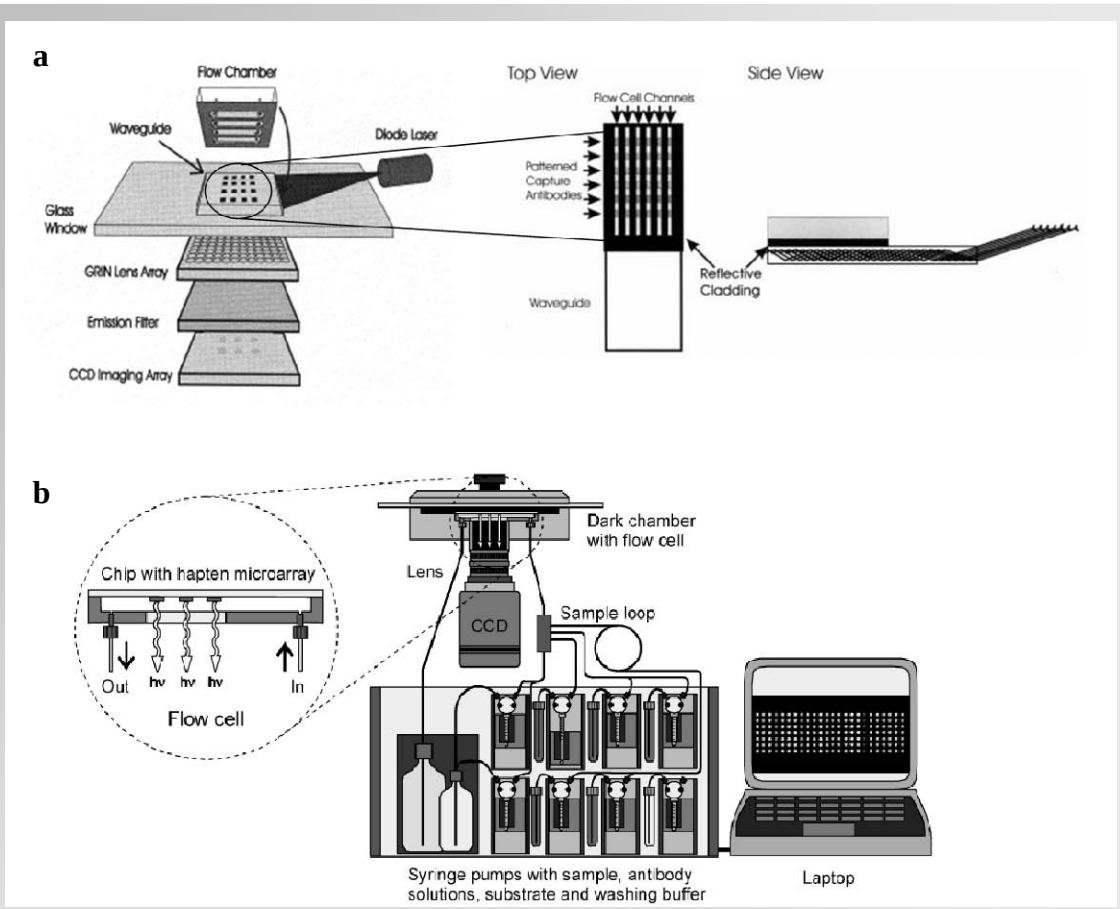

Figure 2.1 Examples of planar array-based technologies. **(a)** The Naval Research Laboratory (NRL) array biosensor based on total internal reflection fluorescence (TIRF). Array sensor optics comprise a patterned glass slide, which is placed on a support and is illuminated by launching 635 nm light from a diode laser into one end, a GRIN lens array, which focus the fluorescent patterns, an emission filter, which rejects unwanted laser light and a Peltier-cooled CCD camera which images the array. Multi-analyte detection is achieved through the "bar-code" approach. Biotinylated capture antibodies are immobilized on the avidin-coated waveguide surface in columns, using a PDMS block with several channels. Adopted with modifications from Feldshtein M. *et al.*[18] **(b)** The parallel affinity sensor array (PASA) is based on a chemiluminescent (CL) read-out of the microarray via the CCD camera and includes a flow cell, and an integrated fluidic system for reagents handling. The disposable chips, microarrayed with haptens or antibodies, are inserted to the flow cell and places in the dark chamber. Free antibody binds to hapten spots and then the secondary antibody, labeled with peroxidase (POD), is introduced. Formation of the immunocomplex is detected by light emission in the presence of a luminol-based POD substrate. The lens and cooled CCD detector are situated beneath the flow cell. Syringe pumps manage the liquid handling, allowing a nearly permanent flow by an alternating action. Adopted with modifications from Knecht B. *et al* [27].

An additional example of a multiplexed platform which utilizes the CL read out is based on 96x4 well plate format [29]. It consists of a 96 well plate, where each well is subdivided into 4 wells. This approach is limited to simultaneous screening of 4 target analytes per samples, but compensates with the amount of samples which can be measured simultaneously in one 96 well plate. Currently it is not automated, however it has the potential to be easily integrated with existing pipetting robots and plate washers.

2.1.3 SPR Biosensors

SPR biosensors do not require the use of reporter elements to generate a signal, which is convenient during assay development and during application by saving labeling steps, washing steps and

time [30]. These sensors are based on the SPR phenomenon which occurs when the light strikes, under certain conditions, an electrically conducting metal film. When the evanescent wave (EW), generated by light, interacts with, and is absorbed by, free electrons in the metal layer, the intensity of the reflected light at a specific angle of reflection is reduced. The incident light angle at which this reduction occurs is called an SPR angle. The SPR angle varies with the refractive index of the dielectric medium (usually buffer) close to the metal film (usually gold). When molecules are immobilized on and/or bound to the gold surface, the SPR angle changes, allowing label-free and real-time monitoring [31]. It is possible to derive an absolute protein concentration on the surface from the SPR response [32], however mostly the relative values of the SPR angle change are used. SPR sensors are used both in a multi-channel and array-based set ups. One of the most popular SPR sensors is the commercially available, 4-flow channel (4FCs)-based, Biacore system. The instrument is fully automated and has a capacity of analyzing up to 192 samples (two 96-wells microtiter plates). The Biacore Q model is dedicated to the qualitative or quantitative determination of analytes in food related products and can be used in combination with specially developed Qflex® Kits. A disadvantage of the Biacore Q is that only one of the four FCs can be used at a time. In the Biacore 3000 model the four FCs can be serially connected and simultaneously monitored. Recently a Biacore 4000 SPR system was launched by GE Healthcare which offers a possibility for simultaneous measurements on 16 spots in 4 FCs. Many methods for food safety and environmental monitoring have been developed on Biacore platforms [33-39]. Alternative eight-channel SPR sensor instrument was developed and used for the detection of low molecular weight endocrine-disrupting compounds [40] and an environmental contaminant in a miniaturized and portable format [41]. Taylor *et al*. reported a custom-build multichannel SPR sensor for the simultaneous detection of four food pathogens [17].

The SPR imaging (iSPR) technology takes SPR analysis a step further, offering much higher multiplexing capabilities. There are several commercial iSPR instruments available, SPRi-Plex™ (Genoptics Bio interactions), ProteOn™ XPR36 (Bio-Rad laboratories), SPRimager®II ARRAY system (GWC Technologies) and IBIS iSPR (IBIS Technologies B.V.). The instruments differ in optics, fluidics, sample handling and available sensor surfaces. All of these factors influence sensor's output, and the choice of the iSPR instrument is usually made accordingly to the application. Recently, a label-free and multiplex detection of antibiotic residues in milk multiplex detection was

demonstrated using the IBIS iSPR system [42]. Seven antibiotics were simultaneously detected in milk, using a competitive immunoassay. The performance of the iSPR biosensor was found to be comparable to other methods and appropriate for several antibiotics monitoring in food. The IBIS iSPR instrument is based on Kretschman geometry and angular modulation of monochromatic (840 nm) plane-polarized light [43, 44]. A 25 mm^2 surface area is illuminated with incident light at different angles which are controlled by a mirror in the range of 8^o (between 66 and 78^o). The sensor chip is placed on top of hemispheric prism (BK7 glass) using refractive index matching oil (n=1.518). The images of the illuminated sensor chip surface are captured by a CCD camera. Light reflectivity is determined from the gray values of the pixels and plotted as a function of the scanning angle. Data acquired from the camera are processed by the software and the responses are expressed as SPR angle shifts in millidegrees. SPR angles are monitored simultaneously on the entire imaged surface using predefined regions of interest (ROIs). Fluidics on the sensor chip surface is handled either by a cuvette or a flow cell. In the flow cell set up, the injected sample is delivered to the sensor surface and pumped back and forth during the interaction. The gold sensor chip surface is usually modified with carboxymethyldextran (CM-dextran). CM-dextran provides a hydrophilic three dimensional matrix for molecular immobilization, improves the sensitivity of the biosensor by increasing the amount of immobilized ligand and reduces nonspecific binding of the sample components to the sensing surface. However, combining optimal conditions for spot formation on the CM-dextran and for immobilization often presents a challenge. When iSPR is applied to concentration measurements, maximal spot load is desired, but if the compound has high molecular weight, only limited number of molecules fit in to the drop that forms the spot on the sensor surface. In such cases, using a spotting technique which utilizes a flow on top of the sensor surface has an advantage over drop depositing techniques [45]. Several portable SPR devices have been developed for field applications [41, 46-50]. However, so far none have demonstrated highly multiplexed analyte detection (>4 analytes simultaneously).

2.1.4 Lateral Flow Devices

Lateral flow devices (LFDs) or dipstick (immuno)assays assays are used for qualitative, semiquantitative and to some extent quantitative monitoring in resource-poor or non-laboratory environments [51]. Applications include fast and easy to apply tests on pathogens, drugs, hormones and metabolites in biomedical, phytosanitary, veterinary, feed/food and environmental settings. Compared with biosensor technology, the LFD technology can be

brought to the market extremely quickly with a relatively small investment. Multiplexing is achieved through the application of multiple test lines. Fenton *et al.* [52] fabricated paper- and nitrocellulose-based LFDs that were shaped in two dimensions by a computer-controlled knife. The resulting structures (e.g. star and candelabra) are spotted with multiple bioreagents to produce multiplex assays in the lateral-flow format. LFD with improved sensitivity (down to the low ng mL^{-1} level) was reported by Mao *et al.* [53]. They developed an LFD for dual protein detection using double test zone containing immunochromatographic strips in combination with gold nanoparticles, that were quantified with a portable electrochemical analyzer.

2.1.5 Novel Planar Arrays

Newly emerging planar technologies include polydiacetylene (PDA) biosensor chips and electrical microarrays. Cheol Hee *et al.* [54] have recently reported development of cross-linked PDA liposome-based chips for multiplex pathogen-detection. PDA supramolecules undergo a color change from blue to red under various stimuli (e.g. temperature, pH and mechanical stress) including binding events that take place on the surface. The red state of the PDA also produces fluorescence. For chip production, the PDA liposomes are arrayed using ethylenediamine as an interlinker on amine-covered glass slides by an array spotter and conjugated with different antibodies. Binding to target bacteria can be monitored by the naked eye, due to chromatic transition, or by measuring the fluorescent out-put (Figure 2.2a). Further research is needed to evaluate the analytical performance of this technology and its applicability to real samples analysis.

Electrical microarrays employ an electrical signal read out from an array of microelectrodes. For example, Elsholz *et al.*[55] described an electrical oligonucleotide microarray for the identification and detection of multiple pathogens via RNA hybridization. The signal was generated by alkaline phosphatase mediated conversion of p-aminophenol to its electrically active phosphate derivative and enhanced by redox cycling (Figure 2.2b). This system was reported to be fast and easy to use and didn't require PCR amplification, but hasn't been applied to food or environmental samples analysis yet. Additional examples to existing analytical microarray systems, which have not been applied to food and environmental monitoring yet, can be found in the review of Seidel *et al.* [56].

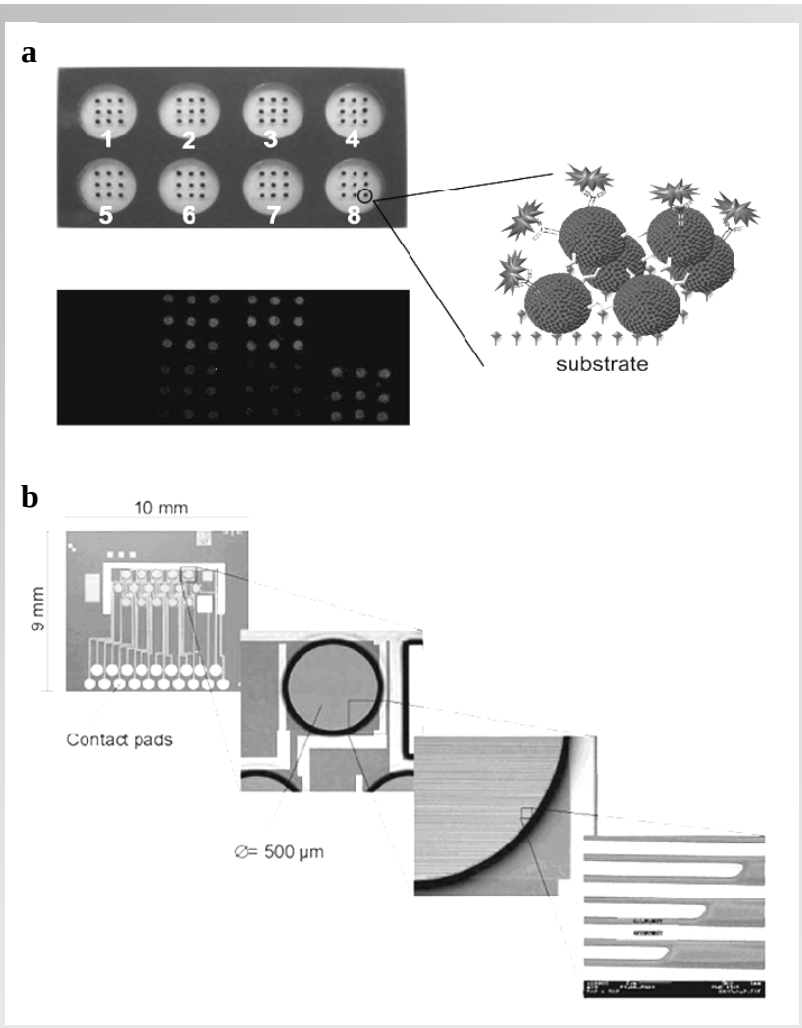

Figure 2.2 Examples for novel multiplexed planar array-based technologies. **(a)** Polydiacetylene (PDA) –based sensor chips. PDA liposomes are arrayed using ethylenediamine as an interlinker on amine-covered glass slides by an array spotter and conjugated with different antibodies. Binding to target bacteria can be monitored by the naked eye, due to chromatic transition, or by measuring the fluorescent output. PDA supramolecules undergo a color change from blue to red under various stimuli (e.g. temperature, pH and mechanical stress) including binding events that take place on the surface. The red state of the PDA also produces fluorescence**.** Adopted with modifications from Cheol Hee P. *et al.*[54]. **(b)** Photograph of electrical biochip microarray design and detailed zoom view. Electrical oligonucleotide microarray is designed for the identification and detection of multiple targets via RNA hybridization. The signal is generated by alkaline phosphatase mediated conversion of p-aminophenol to its electrically active phosphate derivative and enhanced by redox cycling. Adopted with modifications from Elsholz B. *et al.*[55].

2.2 Suspension Array Technologies

2.2.1 Flow Cytometry

Fluorescence-based flow cytometry dates back to the 1960s [57]. Essentially, cells or particles are aligned in a flow stream and optically interrogated. Size, density, and fluorescence at multiple wavelengths can be quantified creating suspension microarrays. A promising and evolving suspension microarray is the Multi Analyte Profiling (xMAP®) technology of Luminex Corporation, which is an open system suitable for assay development. This technology uses small carboxylated polystyrene microspheres (5.6 µm in diameter), which are internally dyed with a red and an infrared fluorophore. By varying the ratio of the two fluorophores, up to 100 different color-coded beads can be distinguished (Figure 2.3a) and each bead set can be coupled to a different biological probe. In combination with flow cytometry, it is possible to simultaneously measure up to 100 different biomolecular interactions in a single well of a 96 well-plate. The carboxylated bead surface allows simple chemical

coupling of capture reagents such as antibodies or drug-protein conjugates. A five-fold increase in multiplexing capabilities can be obtained with the FlexMAP 3D™ platform of Luminex, using a third fluorophor, which offers 500-plex capability and runs three times faster compared with the Luminex 100 or -200 systems. The new Luminex multiplex platform called MagPix™ is also a low-cost, compact, rugged, diagnostic and environmental testing xMAP analyzer. It moves away from a flow cytometry-based system to an instrument based on their already existing magnetic bead array (MagPlex™) analyzed on a magnet in a 2D readout with inexpensive Light Emitting Diodes (LEDs) and a CCD imager. It is expected to be launched in 2010 and these developments will make future multiplexing faster, cheaper and more robust and applicable in the food chain. Using same color-encoded beads, Kim *et al*. [57] developed a robust, simple to fabricate, and very compact microflow cytometer with capabilities for point-of-care and on-site analysis and also relevant to food and environmental diagnostics.

Other microbead-based suspension arrays that are compatible with standard flow cytometers are commercially available from different companies. Bangs Laboratories, Inc. (Fishers, IN, USA) supplies QuantumPlex™ kits that provide a platform for the design of multiplexed suspension arrays on polystyrene microsphere populations in two sizes (4.4 and 5.5 µm) and each with five different intensities of the fluorophor Starfire Red™, which results in 10 populations [58]. They also provide five populations of superparamagnetic microspheres of 6 µm (QuantumPlex™M).

The FlowCytomix™ technology of Bender MedSystems (Vienna, Austria) uses two sets of polystyrol beads with different sizes (4 and 5 µm) and each size consists of bead populations which are differentiated by varying intensities of an internally fluorescent dye. The combination of the two different bead sizes and different internal dye intensities makes it possible to distinguish up to 20 bead sets.

The Cyto-Plex™ carboxylated microspheres of Duke Scientific Corp (Palo Alto, CA, USA) provide up to twelve levels of red fluorescent intensities for analysis of maximal twelve analytes per diameter. These beads are available in 3 different diameters: 4, 5 and 7 µm, which enables the simultaneous quantification of more than 30 analytes within a single sample. Ramirez *et al*. [59] developed a high-throughput flow cytometric system based on the combination of an automated sample handling system and five of the these bead sets (HyperCyt). With this system, samples are aspirated from the microtiter plate and delivered to the flow cytometer for analysis at rates approaching 100 samples per minute. A selection of Cyto-Plex™ beads is also applied in the Sal Plex™ (RnA,

Utrecht, the Netherlands) for the measurement of *Salmonella* antibodies in plasma, serum, meat-drip, eggs and milk. Sal Plex^{TM} beads enable flow cytometric detection of any infection caused by any *Salmonella* serovar belonging to serogroups B, C1, C2, D and E , providing at the same time serogroup information as well. Based on the same beads, Soft Flow, Inc. (St. Louis Park, MN, USA) supplies a fiveplex immunoassay (Fungi-PLEX^{5}) for the simultaneous detection of several mycotoxins in food and feed.

Spherotech, Inc. (Lake Forest, IL, USA) offers additional multiplexed flow cytometry bead assay(SPHERO^{TM}). They supply carboxylated blue, yellow and pink fluorescent Particle Array Kits (PAK) with different intensities and sizes to run 50 assays in the same tube, compatible with most single laser flow cytometers.

The multiplex kits described above are used by companies and clinical laboratories to develop assays for allergy testing, autoimmune diseases, cardiac markers, cytokine detection, endocrine markers, infectious disease markers, isotyping, genotyping, kinase and phosphorylated protein activity, metabolic markers, and tissue typing. Applications in food and environmental analysis are still very limited.

2.2.2 Nanoparticles

Nanoparticles (NPs) research is currently an area of intense scientific investigations, due to a wide variety of potential applications in biomedical, optical, and electronic fields. Nanoparticles are defined as particles having one or more dimensions in the sub-100 nanometer range. In analytical biochemistry, they are used as biosensor response enhancers and as labels in clinical and food diagnostics due to their unique characteristics as the high surface-to-volume ratio and the size-dependent optical or magnetic properties [60, 61].

Yuan *et al.* [62] reported the use of gold NPs (40 nm) for signal enhancement on a mixed self-assembled monolayer (mSAM) sensor surface which resulted in 21.5 fold signal increase and, due to a large reduction in antibody concentration, in a 30 times more sensitive assay for chloramphenicol. The use of gold NPs and other new nanodiagnostic tools (e.g. quantum dots (QDs)) for diagnostic applications promise increased sensitivity, multiplexing capabilities, and reduced costs [63]. The nanometer sized colloidal gold particles are also frequently used as labels in lateral flow tests [64] because of their ability to adhere proteins (e.g. antibodies) and their intense red color due to localized SPR (LSPR) [65]. Localized surface plasmons (LSPs) are charge density oscillations confined to metallic NPs. Excitation of LSPs by light at an incident wavelength where resonance occurs results in strong light scattering, in appearance of intense surface plasmon (SP) absorption bands, and in enhancement of the local electromagnetic field. The frequency (i.e. absorption

maxima or color) and intensity of the SP absorption bands are specific to the type of metal (e.g. gold, silver or platinum), and highly sensitive to the size, shape of the nanostructures and to the changes in the surrounding environment. The fact that the color of metallic NPs depends markedly on the refractive index of the surrounding medium, has been exploited for sensing applications. LSPR sensing is based on a simple optical extinction measurement, is not temperature sensitive, and requires common laboratory equipment. Molecular interaction analysis using gold NPs on a solid transparent substrate (glass) have been reported by several groups. Such an LSPR-based immunosensor was developed for the detection of casein in milk [66] in which anti-casein antibodies were immobilized to gold-capped silica NPs on a glass slide substrate and the binding of casein was monitored by the peak absorbance intensity increments at around 520 nm. Kreuzer *et al*. [67] developed an LSPR-based biosensor for the detection of stanozolol using gold colloids (100 nm), coated with a stanozolol-protein conjugate, chemically sized on an activated glass substrate. Binding of anti-stanozolol antibodies was observed by a shift of the resonance wavelength (with a maximum of 13 nm) with a detection limit of 2.4 nM or 0.7 ng mL^{-1} stanozolol. Prodan E. *et al.* [68] suggested gold-silica core nanoshells for multiplexed assay development. These nanoshells comprise a spherical silica core surrounded by a gold shell of a few nanometers in thickness. Different core/shell ratios result in different optical resonances which are employed for multiplexing. Main advantages offered by the LSPR devices include simplicity of the optical configuration, easy fabrication, great potential for miniaturization, simple handling, low-cost, short assay times, and high sensitivity.

Advances in nanomaterials have produced a new class of fluorescent labels which is more suitable for multiplexed detection by conjugating semiconductor nanocrystals, also known as quantum dots (QDs), with biorecognition molecules [69]. These QDs (2-8 nm) are atom clusters comprising a core, shell, and coating. The core consists of a few hundred to a few thousand atoms of a semiconductor material often composed of atoms from group II-VI (e.g. CdSe, CdTe, CdS, and ZnSe) or group III-V elements (e.g. InP and InAs) in the periodic table. A semiconductor shell (typically zinc sulfide) surrounds and stabilizes the core, improving both the optical and physical properties of the material. An amphiphilic polymer coating then encases the core and shell, providing a water-soluble interface. To reduce nonspecific binding, this amphiphilic coating may be further modified with a functionalized polyethylene glycol (PEG). By varying the size and composition of QDs, the emission wavelength can be tuned. For diagnostic multiplex applications, a set of seven amine-, or carboxyl-derivatized or streptavidin-labeled Qdot® nanocristals (maxima

at: 525, 565, 585, 605, 655, 705 and 800 nm) is commercially available from Invitrogen (www.invitrogen.com). More multicolor optical coding has been achieved by embedding different-sized QDs into polymeric microbeads at precisely controlled ratios [70]. The broad absorption spectra, which is useful for the simultaneous excitation, and the narrow symmetric emission spectra make QDs very well suited to optical multiplexing [61]. Moreover, exitons exhibit a much longer lifetime (up to about 200 nanoseconds) in comparison to excited state of conventional fluorophore. Other advantages of QDs include excellent brightness, negligible photobleaching, fairly high quantum yields, and photostability. These extraordinary fluorescence properties can be attributed to the unique properties of semiconductor materials which produce fluorescence through the formation of excitons or Coulomb-correlated electron-hole pairs, upon absorption of a photon of light.

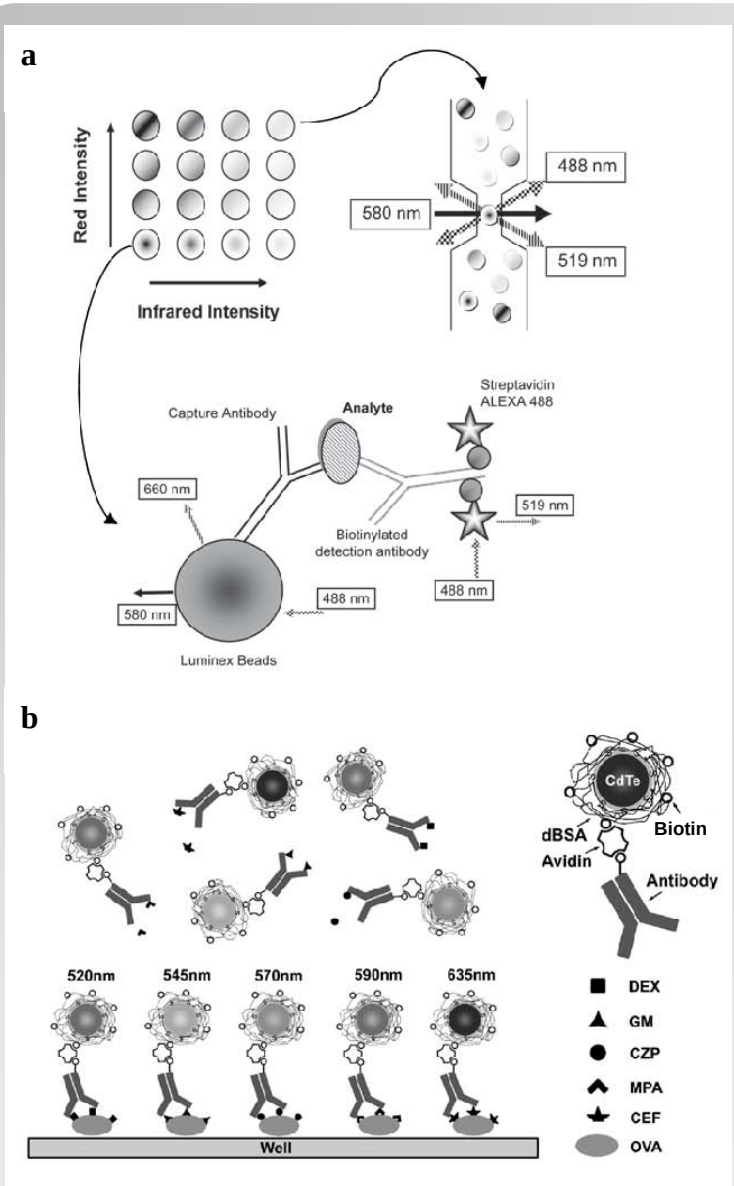

Figure 2.3 Examples of suspension array-based technologies. **(a)** Multiplex microbead immunoassay based on flow cytometry. A set array of beads coded with different ratios of two fluorescent dyes are conjugated with distinct antigens. The analyte is bound to a specific bead and detected with fluorescently-labeled antibody. All the beads are assayed simultaneously in a single tube and subsequently analyzed in the flow cytometer. Adopted with modifications from Krishhan V. *et al.* [71] **(b)** Multiplexed fluorescent immunoassay (FLISA), based on Quantom dots (QDS) for the simultaneous detection of five chemical residues. The microtiter plate is coated with ovalbumin (OVA)- analyte congugates (dexamethason (DEX), gentamicin (GM), clonazepam (CZP), medroxyprogesterone acetate (MPA) ceftiofur (CEF). Five antibodies are then conjugated with the corresponding QDs to establish the indirect competition FLISA. The individual quantitative determinations of five chemical residues are carried out based on the different emission properties of the QDs. Adopted from Peng C. *et al.* [72].

3 Applications

3.1 Foodborne Pathogens

Foodborne pathogens are infectious microorganisms that cause foodborne illnesses through the consumption of contaminated food or water. Even though the prevalence of foodborne illnesses is difficult to determine, it has been estimated that in industrialized countries about 30 % of the population suffer from foodborne illnesses on a yearly basis [73]. Foodborne illnesses are associated with poor hygienic practices, with food providing the link in the fecal-oral route. Major foodborne pathogens include *Salmonella* spp., *Vibrio* spp., *Campylobacter* spp., *Escherichia coli* O157, *Listeria monocytogenes*, and the Norwalk and Norwalk-like viruses. Proper detection methods are vital for food safety maintenance, and in case of food pathogens, a rapid detection of microorganism is necessary to curb outbreaks which can affect large populations. Current microbial pathogen detection methods include standard culturing and biochemical techniques, immunological methods and nucleic acid analysis. Culturing techniques in combination with the biochemical identification are considered to be the most cost-effective, reliable and accurate methods for food pathogen detection. However, they are labour-intensive and time-consuming, requiring around 2-3 days for initial results and another 5-7 days for confirmation. Pathogen identification with the culturing techniques is also complicated by the low number of pathogens in comparison to the surrounding microflora, isolation difficulties from food and culturing of slow-growing or non-cultured pathogens. Immunological methods often provide a faster, less laborious and more specific alternative, depending on the employed antibody and immunoassay format. Most sensitive pathogen detection techniques are based on the polymerase chain reaction (PCR), which in principal can detect a single copy of a target DNA sequence. However, in practice, in many cases it requires pre-concentration and doesn't distinguish between viable and non-viable cells. Implementation of molecular methods such as PCR and ELISA can reduce the detection time to 8 to 48 hours [8, 74]. However, screening each sample for a single pathogen remains time-consuming, labor-intensive and costly. Multiplex technologies offer parallel analysis of several pathogens in a single experimental run, reducing assay time, labor and costs. There are numerous applications of both nucleic acid-based and immunological methods for pathogen detection, in this review we focused on the multiplexed techniques which enable a simultaneous detection of at least two pathogens.

3.1.1 Multiplexed PCR

Multiplexed PCR is a variant of PCR in which two or

more loci are simultaneously amplified in the same reaction using multiple sets of primers [75]. This method has been successfully applied in many areas of nucleic acids analysis including foodborne pathogen detection. Kim *et al.* [76] developed a multiplex PCR assay in a single reaction tube for the simultaneous detection of five pathogenic bacteria: *E. coli* O157:H7, *Staphylococcus aureus, Vibrio parahaemolyticus, L. monocytogenes,* and *Salmonella*. The method required an enrichment procedure (16 hours growth) and provided final results within 24 hours. The five food pathogens were detected from both culture medium and artificially inoculated water, milk, and raw pork meat, with a detection sensitivity ranging from 10 to 100 colony forming units (CFU) g^{-1} at relatively low costs (less than $1 per pathogen). Additional reports on applications of multiplex PCR for foodborne pathogens detection include simultaneous species identification and detection of major serotypes and epidemic clones associated with human listeriosis [77] and the simultaneous detection of *E. coli* O157:H7 and *L. monocytogenes* following a modified method of enrichment and harvesting [77, 78]. A feasibility study was conducted by Kawasaki *et al.* [79] where a multiplex PCR system was applied for the simultaneous detection of *Salmonella* spp., *L. monocytogenes* and *E. coli* O157:H7 in 44 types of food samples, including meat, fish, diary products and frozen food. Each of the three pathogens was successfully detected in most of the food products with a sensitivity of 5 CFU 25 g^{-1}. The method required 20 hours of a non-selective enrichment step in No 17 medium, to allow recovery of injured cells and the simultaneous enrichment of the three pathogens. To reduce nonspecific PCR products, due to the amplification of unexpected primer binding sites, nested PCR can be applied. It uses two sets of primers, the first to amplify the target sequence and the second set of primers ”the nested primers” bind inside the first PCR product. Nested multiplex PCR (nmPCR) was applied to evaluate the geographic impact of contamination by *Salmonella* in estuarine water and sediments [80]. The presence of *Salmonella* spp., including their non-culturable form, was based on flagella gene (flicC) amplification, adapted for the detection of the major serovars, without any cultivation steps. The detection limit of the assay was estimated to be 1 CFU in deionized water, and 4–5 CFU in estuarine water seeded with *Salmonella*.

PCR-amplified DNA is traditionally analyzed by the agarose gel electrophoresis, however, it can also be coupled to other techniques. For example, multiplex PCR coupled to capillary gel electrophoresis (CGE) offers increased sensitivity, robustness, automation and parallelization [81-83]. Alarcon *et al.* [84] described the simultaneous detection of *S. aureus*, *L. monocytogenes* and *Salmonella* spp. by multiplex PCR followed by CGE with laser

induced fluorescence (LIF) detection. The use of multiplex-PCR-CGE-LIF significantly improved the levels of the three pathogens detection, allowing 5.7 x 10^2, 7.9 x 10^2 and 2.6 x 10^3 CFU mL^{-1} in inoculated raw beef samples without enrichment and 6, 8 and 26 CFU mL^{-1} after a 6 hours enrichment step. PCR products can be also analysed using DNA microarrays [85-88]. For example, Gonzales *et al.* [89] applied multiplex PCR coupled to DNA microarray analysis for marine fish pathogen detection. Additional examples for the application of multiplex PCR in food safety include the simultaneous detection of *Salmonella* spp. and *E. coli* O157:H7 [90], *Salmonella* spp. and *Shigella* spp. in mussels [91], *C. jejuni*, *Salmonella* spp., and *E. coli* O157:H7 in a raw and ready-to-eat food products [92], *E. coli, Salmonella typhimurium, V. vulnificus, V. cholerae, and V. parahaemolyticus* in shellfish [93], *L. monocytogenes* and *Salmonella* in cooked ham [94] and six bacterial pathogens in marine waters [95]. The highest multiplex capacity so far, for the simultaneous identification of eight foodborne pathogens (*Escherichia coli, Clostridium perfringens, C. jejuni, Salmonella enterica, L. monocytogenes, V. parahaemolyticus, S. aureus, and Bacillus cereus),* uses capillary electrophoresis and single-strand conformation polymorphism for analysis of the PCR amplified fragments [83]. Technical aspects on the use of multiplex PCR to detect and differentiate food- and beverage-associated microorganisms can be found in the review by Settanni L. *et al.* [96].

There are several challenges for food pathogen detection with multiplexed PCR approach. For instance, the recovery of a small number of cells from a complex food matrix is difficult due to high background microflora and possible unknown PCR inhibitors, and thus pre-detection enrichment of microbial contaminants is usually necessary for a successful PCR amplification. Additionally, using multiplex primers may result in biased PCR amplification, favouring particular template-primer combination over others, causing misrepresentation of the unfavoured target. Careful design of the primers together with modification of pre-detection growing conditions are needed to prevent this problem [78]. Improved sensitivity and specificity can be obtained by using the nested version of PCR, as described above [80]. In order to obtain information on viable cells, reverse transcriptase-PCR (RT-PCR) [97] or bacteriophages [98] could be utilized for pathogens detection [97-99]. Multiplex quantitative real-time PCR is also limited by the number of available fluorescent probes, which are needed for detection, and/or differences in melting temperatures [99]. In comparison to culturing techniques, multiplex PCR offers simultaneous detection of multiple pathogens in one reaction tube in a rather short time, however, its application for routine screening thus far is rather limited due to inherent

labour intensiveness, high costs and requirement for highly trained personnel and a dedicated lab environment.

3.1.2 Planar Arrays

Research in the field of biosensors is focused on the development of rapid, sensitive, simplified and recently also on multi-analyte techniques, as alternatives to traditional platforms for pathogen detection. Most of the biosensors incorporate the same bioassay principals as traditional methods with transducers (optical, electrochemical, etc) in a novel, usually miniaturized, integrated analytical device. The NRL array biosensor, based on TIRF, in combination with a sandwich immunoassay was applied for individual detection and quantification of *Shigella* and *Campylobacter* spp. in ground turkey, chicken carcass wash, milk, lettuce leaf and river water [25]. The authors reported detection limits of 4.9×10^4 and 9.7×10^2 CFU mL^{-1} (in buffer) for *Shigella dysenteriae* and *C. jejuni*, respectively. Ground turkey and buffered milk were found to cause the greatest matrix effect on the dose-response curve, lowering the bioassay's sensitivity. This multianalyte immunoassay system was also successfully implemented for the simultaneous detection of *S. typhimurium*, *Shigella dysenteriae* and *C. jejuni* spiked into chicken carcass wash. The assay time was reported to be 25 minutes; however, to reach the necessary sensitivity for effective use by the food industry, this system should be coupled to an enrichment step prior to analysis. The use of antimicrobial peptides for the detection of *E. coli* and *Salmonella* has been demonstrated in this system as well [100, 101].

Recently, Hee *et al.* [54] reported a polydiacetylene (PDA) liposome-based biosensor for the multiplex pathogen detection. Interlinked PDA liposomes were spotted on amine covered glass and covered with perforated silicone to create 8 microwells, each containing 9 spots (0.5 mm in diameter). PDA liposomes in each well were conjugated with an antibody against a different pathogen, leaving 2 wells unconjugated for control. The binding of the pathogen to the liposomes was detected directly, due to the PDA colour change from blue to red and by the fluorescence of the red state. A proof of concept was demonstrated using two mixtures, one containing *Cryptosporidium parvum* and *E. coli O-157* and another containing *Giardia lamblia, S. typhimurium* and *Encephalitozoon intestinalis* at concentrations of 10^6 CFU mL^{-1}. The presence of pathogens could be detected either qualitatively by the chromatic transition of PDA to red or quantitatively by the fluorescence signal, within 30 minutes.

A 96-well microtiter plate–based antibody microarray was developed by Gehring *et al.* [102]. The sample was statically incubated in avidine-coated wells where biotinylated

capture antibodies were spotted. The presence of captured analyte was reported with a fluorophore-conjugated antibody. *E. coli* O157:H7 and *S. typhimuruim* were detected at concentrations of 10^6 and 10^7 CFU mL^{-1}, respectively, within 2.5 hours in buffer and in ground beef. Alongside, chicken IgG was detected at ng mL^{-1} levels, as a model to a proteinaceous toxin. The sensitivity achieved with this method was less than the sensitivity of the NRL array biosensor [25]. Microarrays in a multiwell plate format offer automatic sample handling during multiple-steps, including sample enrichment and cleaning, enabling screening of large sample sets.

A flow-through microarray coupled with a CL read-out was described by Wolter *et al.* [103]. With this method, multiple bacteria were simultaneously detected in water samples within 13 minutes. CL-based detection enabled optical read-out of the microarray with high sensitivity and without external light source. Polyethylene glycol modified glass was used as a support platform for a sandwich immunoassay for the parallel detection of *E. coli* O157:H7, *S. typhimurium* and *Legionella pneumophila,* with limits of detection of 3 x 10^3, 3 x 10^6 and 1 x 10^5 cells mL^{-1}, respectively. The binding of the bacteria to capture antibodies was reported by specific antibodies labelled with biotin and horseradish peroxidase-streptavidine conjugate, which produced chemiluminescence in the presence of luminol and hydrogen peroxide. Karsunke *et al.* [104] modified this method by developing a disposable plastic multichannel version, using an acrylonitrile-butadiene-styrene copolymer. The chip contained six flow-through microchannels, which enabled calibration and measurement in one experiment, reducing the total assay time to 18 minutes. For monitoring drinking water supply, both systems should be used after bacteria enrichment steps, such as microfiltration or immunomagnetic concentration. Immunoassays combined with a CL read-out have been implemented also in a microtiter plate format. Magliulo *et al.* [29] developed a CL-EIA for multiplex detection of *E. coli* O157:H7, *S. typhimurium, Yersinia enterocolitica* and *L. monocytogenes.* Using a new polystyrene plate design, where each of the 96-wells contained 4 subwells at the bottom, the four bacteria were simultaneously detected in meat and faecal samples with 10^4-10^5 CFU mL^{-1} sensitivity. Another electrochemistry-based system has been reported as a tool for detection of viable *E. coli* subspecies where screen-printed disposable electrode arrays were utilized to monitor respiratory activity of bound cells [105]. Pathogenic bacteria were isolated from pure liquid cultures and agar plates by binding to lectin-modified membranes, which were later on layered over individual screen-printed carbon electrodes of the sensor array. Quantities of bound cells and specific patterns of chronocoulometric signals for four *E. coli*

subspecies was obtained through electrochemical oxidation of ferrocyanide. Total analysis time was reported to be 40 minutes. Application of this technology to complex sample matrices will probably require sample pre-treatment in order to remove matrix components which interact with lectins [105]. A portable point-of–care device, also based on electrochemical detection, was also reported for the simultaneous measurements of *E. coli* and *B. subtilis* DNA, utilizing a silicon glass-based micro chamber [106].

Recent developments in SPR towards multi-analyte platforms currently enable label-free read-out of binding events on the microarray surface. Taylor *et al.* [17] reported the application of an eight-channel SPR sensor for quantitative and simultaneous detection of *E. coli* O-157*, Salmonella choleraesuis* typhimurium*, L. monocytogenes and C. jejuni* in buffer and in apple juice with sensitivities ranging from 3.4×10^3 to 1.2×10^5 CFU mL^{-1}. A sandwich immunoassay was performed, in order to amplify the direct response obtained with bacteria binding to the sensor chip surface. The LODs obtained with this sensor were comparable to those obtained with the NRL array biosensor, however the assay time was longer (approximately 100 minutes in the SPR sensor) [25]. A direct immunoassay, using monoclonal antibodies spotted on Protein G modified gold sensor chip, was reported for the detection of *E.coli, S. typhimurium, L. pneumophila,* and *Y. enterocolitica* [107]. SPR-based biosensors were also used for serological monitoring of the pathogens in farmed animals. For example, Jongerius-Gortemaker *et al.* [34] demonstrated detection of antibodies against *Salmonella* in chicken serum, suggesting this approach for detecting past or present infections with a range of pathogens in animals. A comparable fast assay (testing in minutes) was developed for the detection of antibodies directed to *Salmonella* sero-groups B and D in porcine blood sera in a routine setting by Achterberg, *et al.* [33].

Label-free detection of multiple bacteria was also realized using a light-scattering sensor [108]. This system is capable of real-time multiple pathogen detection in a Petri dish format without any kind of labelling. The identification is based on unique light scattering fingerprints of each colony forming bacteria, resulting from variable amounts of bacterially produced exopolysaccharide. The authors reported detection and identification of *E. coli, Salmonella, Listeria, Staphylococus* and *Vibrio* with a sensitivity of 1 CFU in 25 g of sample and almost 100 % of specificity within 24 hours and in the presence of background flora. Implementation of biosensors to multiplexed food pathogen analysis reduces analysis time from hours to minutes. However, only a small number of studies reported highly sensitive devices, which enable pathogens detection without the pre-enrichment steps.

3.1.3 Beads and Nanoparticles

The use of beads with varying properties in bioassays offers many multiplexing possibilities. Here are the examples of such systems applied to foodborne pathogen detection. The Luminex xMAP platform was evaluated for multiplexed detection of DNA or proteins from common bacterial pathogens by Dunbar *et al.* [109]. Amplified target DNA sequences were detected with a sensitivity of 10^3 genome copies for *E. coli, L. monocytogenes, C. jejuni* and 10^5 for *Samonella*, within 40 minutes following amplification. Species-specific immunoassays for bacterial antigens, on the same platform, allowed detection of a few to several hundred organisms per mL, within 3 hours following the sample preparation. Both DNA and protein-based multi-pathogen analysis in the Luminex xMAP system provided a rapid and cost-effective alternative to traditional methods.

A multiplexed bead-based mesofluidic system (BMS) was developed for the simultaneous detection of eight major foodborne pathogens: *Salmonella enterica, S. aureus, L. monocytogenes, V. parahaemolyticus, Shigella sonnei, Enterobacter sakazakii, E. coli* O157:H7 and *C. jejuni* [110]. Glass microbeads, coated with specific nucleotide probes, were arranged in polydimethylsiloxane (PDMS) microchannels in a predetermined order. Fluorescently labelled PCR products of pathogenic amplicons were infused into the microchannels, where they were captured by corresponding probes. The mesofluidic chip was scanned and fluorescent intensities were determined for each group of beads. Detection limits obtained for the eight tested pathogens from pure cultures were in the range between 5×10^2 and 6×10^3 CFU mL^{-1}. Analytical performance of the BMS system in food matrixes was evaluated by screening 184 endogenously infected food samples, including eggs, pork, chicken, shellfish, ice cream and milk powder. The tested pathogens were correctly detected and identified in all food samples and were consistent with the results obtained with culture and biochemical identification methods. All operations were controlled with a peristaltic pump, allowing simple manipulation of the beads as well as injection, hybridization and washing steps handling. The use of microchannels reduced the sample volume required for analysis, prevented evaporation from the chip surface and cross contamination. Analysis with the BMS system could be as fast as 30 minutes and offered a powerful new platform for foodborne pathogen analysis [110].

Fluorescent nanoparticles (NPs) incorporating different ratios of three dyes, were suggested for the simultaneous detection of multiple bacteria by Wang e*t al.* [111]. The proof of concept was demonstrated by coating the NPs with polyclonal antibodies against *E. coli*,

S. typhimurium and *S. aureus* via PEG-streptavidin-biotin-IgG conjugation chemistry and incubating with the target bacteria for 30 minutes. Following washing and centrifugation steps, confocal imaging of the target bacteria showed specific coverage with the fluorescent NPs. Despite promising multiplexing possibilities, the analytical capabilities of this method are yet to be evaluated. Another study reported the use of commercially available fluorescent semiconductor quantum dots (QDs) for parallel detection of *E. coli* and *S.* typhimurium coupled with immuno-magnetic bead separation [112]. QDs allow single wavelength excitation with different emission peaks, providing a platform for multicolour imaging. The authors reported a detection limit of approximately 10^4 CFU mL^{-1}, with a needed total assay time of 2 hours. Multiplexing capacity in this assay was rather low (up to 4 species) due to limited availability of commercial QDs. Even though QDs offer many advantages over conventional fluorophores, such as improved photostability and brightness, their synthesis is considered to be difficult, and commercial variants are limited and expensive.

As an alternative to fluorescently labelled NPs, gold nanorod probes-based pathogen monitoring was proposed, applying LSPR detection principle [113]. Optical properties of gold nanorods are shape dependent and are affected by the changes in the dielectric constant in the vicinity of the nanorod surface, phenomenon which is also known as LSPR. The elongated shape of the particles provides higher sensitivity to the local dielectric environment in comparison to spherical nanoparticles in the same size. Gold nanorods can be easily fabricated at different aspect ratios offering multiplexing possibilities. Simultaneous detection of *E. coli* and *S. typhimurium* was achieved using amine-modified gold nanorods with different aspect ratios, coated with polyclonal antibodies against the two pathogens [113]. Within 30 minutes, the target pathogens were detected simultaneously at concentrations less then 10^2 CFU mL^{-1} . Superparamagnetic particles were also employed for multianalyte detection of food pathogens. Koets *et al.* [114], reported a giant magneto resistance (GMR)– based biosensor for the simultaneous detection of four antibiotic resistant genes of *Salmonella* with picomolar sensitivity.

To summarize on multiplex systems for foodborne pathogen detection developed so far, biosensors offer a promising alternative for the conventional molecular and culture techniques. Biosensors demonstrated the shortest measurement times (minutes) with high multiplexing capabilities and comparable sensitivities to most molecular methods. However, this cutback in detection time is only beneficial when no enrichment steps are needed, and thus higher sensitivities should be reached, pushing bacteria detection limits

towards a single organism. NPs integration with the biosensor platforms may provide the necessary enhancement in sensitivity. NPs amplify biomolecular recognition events due to their large surface-volume ratio, which increases the amount of the molecules immobilized onto the surface and maximises binding events. As a result, NPs offer enormous signal enhancement when coupled with sensitive optical or chemical transducers, providing the basis for ultrasensitive detection. So far, the pre-enrichment steps seemed to be necessary for most of the reported techniques. The choice of the pre-enrichment step greatly influences the total analysis time. For instance, if enrichment by culturing is applied, the assay time increases by days. However, if immunomagnetic enrichment is used for pre-concentration, the analysis time is increased merely by a couple of hours [115]. Most likely, conventional microbiology in routine food safety and environment monitoring will not be completely replaced. However, array-based technologies and multiplexed PCRs have already become common techniques in today's laboratories and portable biosensors will probably dominate the on-site pathogen detection in the future.

3.2 Food Allergens and Proteinaceous Adulterants

Proteins which are related to food contaminants include mainly allergens and markers for the detection of food adulteration. Adulteration is any undeclared substitution or addition designed to enhance the economic value of the food product. Generally, it features omission or substitution of valuable components with cheaper alternatives and/or concealment of intrinsic low quality or product damage [116]. For instance, soy proteins are considered to be major potential adulterants in milk products, due to the commercial availability of several preparations (flours, textured flours, protein concentrates, isolates and hydrolysates) at low price. Other possible sources for adulterants include wheat gluten, maize, pea, bean, rice and potato proteins as well as gelatin, blood plasma, egg and fish proteins [36]. Undeclared animal species in meat and fish products are other examples of common food adulterations [117]. The outcome of most food adulteration cases is economical, resulting in consumers fraud by selling the products for higher value than they are worth. Additional concerns include possible allergic reactions to the adulterants and offending religious beliefs. Food allergens can be adulterants as well, if the product is improperly labeled. For consumer protection, legislation requiring a mandatory declaration of allergenic foods has been put into place both in the EU and in the USA [118, 119]. Since the prevalence of food allergy continues to rise, especially in industrialized countries, where 2 % of the adult populations

and 5-8 % of children are affected, the quantitative detection of allergens in the food chain became a strategic health objective [120]. Certain forms of adulteration can be also unintentional, for example, cross-contamination due to shared manufacturing and storing facilities. Therefore, adequate monitoring techniques are of an interest to both legal authorities and to the food industry.

Early methods for multiplex protein detection relied on electrophoretic separation techniques often combined with the more specific immunodetection (Counter-immunoelectrophoresis, Western blots (immunoblots), and radioimmunoprecipitation) [121]. With these methods, multiple proteins could be specifically detected using antibodies after the separation step. These procedures, in a standard format, are lengthy and cannot be performed outside of specialized laboratories. For many proteins, the fastest and the easiest detection method was proved to be the enzyme-linked immunosorbent assay (ELISA). ELISAs have been successfully implemented for specific protein detection, also in complex mixtures, but are most commonly used in a singleplex assay format. The next generation of protein detection techniques is based on miniaturized planar and suspension arrays and enables multiplexed protein analysis. Most of the currently available multiplex immunoassays for proteins have been designed for clinical diagnostics for the quantification of multiple protein biomarkers (e.g. antibodies and cytokines (proteins and peptides secreted by specific cells of the immune response)), because their concentrations provide information about numerous diseases and inflammatory conditions [122, 123].

When dealing with diagnostics for allergens, a large panel of well-established indirect methods exist which do not detect the allergen but the reactions of the allergic individuals. Renault *et al.* [124] reviewed past and current allergy diagnosis in which they concluded that miniaturized (recombinant) allergen micorarrays became important tools in the determination of allergen-specific IgE, particularly in large screening programs, due to their simplicity, low sample volume, high-throughput capacity and flexibility. The “All-Diet” approach, by creating an array of crude and purified extracts of foods found in the British diet, is one of their described future developments. Direct food allergen monitoring techniques include protein-based methods in various formats (immunoblotting, enzyme or radio-allergosorbent test, rocket-immunoelectrophoresis, etc.) and DNA-based methods [125]. However, the most commonly and routinely used immunochemical method for food allergen detection is the ELISA for each single allergen. As an exception, a multi-allergen ELISA in the competitive indirect format has been described for the simultaneous determination of peanut and several tree nut allergens in chocolate with limits of detection

below 1 μg g^{-1} protein for each allergenic food [12]. They assembled multi-allergen microtiter plates by combining 8-well strips coated with proteins from each of the five allergenic foods. A multiplex reverse dot blot enzyme immunoassay system, using spots of egg yolk antibodies (IgY) specific for different allergens on a strip of polyester cloth in combination with allergen specific enzyme-labeled antibodies, has also been developed for the multiple detection of allergens with an LOD of 0.1 μg g^{-1} for peanut allergens in various food, and for hazelnut and Brazil nut allergens in chocolate ice cream [126]. A flow channel-based SPR biosensor (Biacore Q) was used to develop both direct and sandwich singleplex immunoassays for the detection of proteins from milk, egg, hazelnut, peanut, shellfish, and sesame in food samples with detection levels down to 1-12.5 μg g^{-1} [35]. They considered the sensitivity of the biosensor technique comparable to the most sensitive ELISAs with short analysis time, normally less than 10 min per sample, as the main advantage. Other advantages are the label-free detection, the high degree of automation and the possibility for the simultaneous detection of several analytes in the same extract by serial analysis in different flow channels. The application of an array biosensor for fluorescent sandwich immunoassays on the surface of a planar waveguide [127] was demonstrated for the detection of ovalbumin as an indicator of egg contamination within 16 min and with limits of detection of 25 pg mL^{-1} in buffer and 1.3 ng mL^{-1} in ten times diluted non-egg pasta extract. Optical Resonance-Enhanced Absorption (REA)-based near-field biosensor immunoassay was proposed as a novel platform for allergen detection by Maier *et al.* [128]. In this study, gold NPs were used as probes for signal generation in a distance-dependent interferometric setup in a planar chip format. Aluminium discs (13 mm diameter) coated with poly(styrene-methyl methacrylate) and a specific polyclonal antibody were used to detect ovalbumin and ovomucoid with a sensitivity of 1 ng mL^{-1}. Main advantage of this approach is that the signal is visible to the naked eye and thus has minimal technical requirements. For the rapid and simultaneous detection of several allergens, Rebe Raz *et al.,* [129] constructed a reusable antibody microarray directed against twelve major food allergens on a hydrogel-coated SPR chip and applied it to label-free and direct allergen detection in food using an angle scanning imaging SPR (iSPR) system. Each measurement cycle (including chip stabilization, interaction with the sample and chip regeneration) produced quantitative data on the concentration of twelve allergens within 12 minutes. The sensitivity of the on-chip allergen detection, expressed in limit of detection (LOD) and limit of quantification (LOQ) of allergen protein in the food sample, was found to be in the low μg g^{-1} range both for cookies and dark chocolates, which is

adequately compatible with food allergens analysis and comparable to most commercially available ELISAs. This approach offers a powerful analytical alternative to existing methods and opens the door to automated and high-throughput allergen analysis. Since food processing can alter properties of the allergens, DNA–based techniques are used for allergen detection as well [125, 130]. Recently, a single dye (SYBR® Green I) multiplex RT-PCR was reported by Pafundo *et al.* [131] for the simultaneous detection of six allergens. The multiplex was achieved via judicious choice of primers which generated amplicons with different melting temperatures. The method was tested in different food matrices and showed sensitivities in the range of 1 to 100 ppm (mg kg-1).

Multiplex bioassays for food adulterants detection are still scarce. Matsunaga et al. [132] developed a multiplex PCR for the simultaneous qualitative detection of six meat species in cooked meat. A multiplex PCR was also described for the detection of ruminant, poultry, fish and pork materials [133]. Several food products were screened, including commercial meals, pet food and baby food with detection limits of 0.004 % fish and 0.002 % for ruminants, poultry and pork. Detection of animal DNA by multiplexed PCR may be especially useful for food products which are submitted to denaturing technologies. Recently, also a multiplex RT-PCR detection was reported by Zeng *et al.* [134]. By targeting cytochrome b genes of mitochondrial DNA, three kinds of animal derived materials (bovine, goat and sheep) were simultaneously detected. The developed method was more sensitive than routine PCR and didn't require electrophoresis or restriction digestion. Two multiplex systems were developed by Haasnoot *et al.* [36,135] for the detection of plant proteins in milk powders. First they employed a direct biosensor immunoassay, developed on the SPR Biacore 3000 platform, using affinity purified polyclonal antibodies immobilized on the sensor chip surface [36]. With this method, soy, pea and soluble wheat proteins were simultaneously detected in milk powders, within 5 minutes and with limits of detection lower than 0.1 % plant protein in the total milk protein content. Their second multiplex system used the fluorescent microsphere-based flow cytometric competitive immunoassay. The described triplex competitive immunoassay was realized by coupling soluble wheat proteins and proteins from soy and pea to three different microsphere sets. A mixture of these microsphere sets was incubated with a mixture of three affinity-purified polyclonal antibodies raised against these proteins and labeled with a fluorophore. The fluorescence intensities on the microspheres were directly measured with the Luminex flow cytometer without any washing steps. The sensitivities of the three assays were determined as 0.5-0.6 µg mL^{-1} at 50 % binding inhibition. This multiplex assay was

easy to extend with other assays by using commercially available non-affinity-purified polyclonal antisera in combination with a fluorescent (PE)-labeled secondary antibody. This was demonstrated in our laboratory by the addition of the maize immunoassay to the triplex immunoassay. Other suggested applications for this multiplex detection of vegetable proteins might be adulterations of meats and sausages.

This microsphere-based technology was also applied for the detection of Cry1Ab protein in genetically modified maize with limits of detection and quantification (weight of genetically modified organism (GMO)/weight) of 0.018 and 0.054 %, respectively, and was described as the first application of a quantitative high-throughput immunoassay in GMO analysis with multiplex options by Fantozzi *et al.* [136]. Because the expression and translation of genes can be low, sensitivity and reliable quantitation of GMO related proteins is often a problem with immunoassays [137]. Improved sensitivity may be achieved for example by combining the immunoassay with PCR [138]. However, GMO testing is mainly based on nucleic acids analysis and have been extensively reviewed by Elenis *et al.* [139]. Recently, Bremer *et al.* [140] reported the application of the microsphere-based technology for the indirect detection of recombinant bovine somatotropins (rbST) via changes in multiple rbST-dependent biomarkers in cow serum. rbST enhances growth and lactating performances of livestock, however its use is banned in the EU [141, 142]. The simultaneous detection of total insulin like growth factor 1 (IGF1) and one of its binding proteins was demonstrated with sensitivities in the low ng mL^{-1} range. Further multiplexing with additional biomarkers will allow achieving a detailed serum biomarker profile and an efficient screening for rbST abuse in food-production animals.

3.3 Toxins

Toxins can be defined as substances that are synthesized by plant species, animals, or by micro-organisms and produce adverse health effects. Many of the toxins from plants and micro-organisms (bacteria, fungi and phytoplankton) are associated with food-borne illnesses. These natural toxins range in size from a few hundred daltons to large proteins of several hundred kilodaltons. Due to the variety of structures of these toxins, it is impossible to use one standard technique for analysis and/or detection and the variety of methods to detect these toxins include for instance high pressure liquid chromatography (HPLC), mass spectrometry (MS) and ELISA. These procedures are time-consuming, labor intensive, costly, and, in the case of ELISA, usually test for one compound at a time. Many HPLC-MS methods for the detection of multiple mycotoxins within a single chromatographic run were described in the review of Turner *et al.*[143], nonetheless they concluded that future trends should focus

on alternative rapid assays and tools to measure multiple toxins from a single matrix. Most of the rapid assays are based on immunoassays and for small compounds, such as mycotoxins, they are generally constructed as competitive assays. For mycotoxins, there are many commercially available immunoassays in a 96 well plate format ELISAs or in LFDs [144]. However, they usually test for one compound at a time. Miniaturization of optical and fluid-handling components has opened up possibilities for further reducing the size of traditional immunoassays. One of the driving forces behind miniaturization is the desire to test for multiple toxins simultaneously.

Microchannel SPR-based immunoassays have been described for many mycotoxins [144]. Most of these methods used a single ligand format in which a toxin or a toxin-protein conjugate is immobilized on the sensor surface and the binding of toxin-specific antibodies is measured. Recently, such a rapid SPR screening assay has been described for the combined detection of T2- and HT2-toxin in naturally contaminated cereals and maize-based baby food using a sensor chip coated with an HT2-toxin derivative and an HT2-toxin monoclonal antibody with high cross-reactivity to T2-toxin [145]. An application of a SPR-based biosensor (Biacore 2000) was described for the simultaneous detection of four mycotoxins [39]. Aflatoxin B_1 (AFB_1), zearalenone, deoxynivalenol (DON) and fumonisin B_1 (FB_1) were simultaneously detected by using four serial connected flow channels coated with the different mycotoxins in combination with a mixture of antibodies. These *Fusarium* and *Aspergillus* toxins could be detected in the analytically relevant range from 0.2 to 50 ng g^{-1} using a simple extraction and clean-up procedure within a time frame of 25 min.

A microarray of immobilized antigens on a plastic probe tray in combination with polyclonal antibodies and an enzyme-labeled second antibody was described for the simultaneous detection of AFB_1 and FB_1 with detection limits in standard solutions of 3 and 43 ng mL^{-1}, respectively [146].

Applications of the NRL array biosensor for the rapid and simultaneous detection of multiple toxins was reviewed by Taitt *et al.* [147]. It has been used for the detection of ochratoxin A (OTA), DON and AFB_1 individually and in combinations in various food matrices using the competitive assay format with mycotoxin derivatives immobilized onto the waveguide and cyanine 5 (Cy5)-labeled antibodies. Additionaly, it was applied for the detection of two large protein toxins (botulinum toxoid A (BotA) and staphylococcal enterotoxin B (SEB)) in the sandwich immunoassay format in various food matrices.

Cholera toxin and ricin have also been detected with this system with detection limits as low as 1.6 and 8 ng mL^{-1}.

Goldman *et al.* [148] prepared bioinorganic conjugates of highly luminescent nanocrystals (CdSe-ZnS core-shell QDs) and antibodies to develop a fourplex immunoassay in a microtiter plate for the simultaneous detection of four toxins (cholera toxin, ricin, shiga-like toxin 1 and SEB) in a single well using QDs with emission maximums of 510, 555, 590 and 610 nm.

Pauly *et al.* [149] developed a multiplexed immunoassay for the simultaneous quantification of five bacterial and plant toxins in complex matrices using the xMAP technology. Sandwich immunoassays were combined for the proteotoxins ricin, abrin, botulinum neurotoxins type A and B and SEB and excellent sensitivities ranging between 2 and 546 ng L^{-1} were obtained in a minimal sample volume of 50 µl. Advancing the existing bead array technology, the novel magnetic and fluorescent microbeads were introduced for an enrichment step, which further increased the sensitivity of the assay to 0.3-85 ng L^{-1}, enabling analysis in a 500 µL sample volume. The method was successfully applied for the simultaneous identification of the target toxins in complex food matrices like milk, baby food and yoghurt. In our group, this magnetic bead-based technology was used to develop a multiplexed competitive immunoassay for the detection of several mycotoxins in buffer. The application of this multiplexed assay to measurements of the target toxins in food and feed extracts is an ongoing work. There is a commercially available fiveplex immunoassay (Fungi-PLEX^5, Soft Flow, Inc.) for the simultaneous detection of AFB_1, OTA, FB_1, T2-toxin and ZEA , based on microspheres produced by a different company (Dukes Scientific Corp.). An additional example of the application of the xMAP technology for the development of a multiplexed bioassay was reported by Wang *et al.* [150].They used the non-magnetic beads for the simultaneous detection of five biohazardous agents: *B. anthracis* spore, *Y. pestis*, SARS-CoV, SEB and ricin, in powder samples. This xMAP-based multiplexed bioassay demonstrated high reproducibility and a higher sensitivity than ELISA. Based on the same beads, Kim *et al.* [57] developed a robust, simple to fabricate, and very compact novel microflow cytometer. It was applied for the detection of bacteria and toxins in the sandwich immunoassay format. The respective limits of detection for the bacteria *E. coli*, *Listeria* and *Salmonella* were found to be 10^3, 10^5, and 10^4 CFU mL^{-1}, respectively and for cholera toxin, SEB, and ricin 1.6, 0.064 and 1.6 ng mL^{-1}, respectively, displaying similar sensitivity to the commercial xMAP system.

Another approach for the simultaneous detection of SEB and immunodominant antigen A homologue of *S. epidermidis* uses an electrical protein array chip technology [151]. This procedure is based on an enzyme-linked sandwich immunoassay in which the detection is achieved by measuring the electrical current generated by redox recycling of an enzymatically released substance. The toxins could be detected in milk and urine in a concentration of 1 ng mL^{-1} within less than 23 minutes.

Mak *et al*. [152] combined the specificity of immunoassays with the sensitivity and simplicity of magnetic detection to develop a novel multiplex magnetic nanotag-based detection platform for mycotoxins that functions on a sub-picomolar concentration level. Unlike fluorescent labels, magnetic nanotags (MNTs) can be detected with inexpensive giant magnetoresistive (GMR) sensors such as spin-valve sensors. They reported simultaneous detection of AFB_1, zearalenone and HT2-toxin in the sandwich immunoassay format with pg mL^{-1} detection limits.

Marine toxins are currently monitored by means of a bioassay based on whole living organisms which requires the use of many mice, and hence poses a technical and ethical problem in many countries. The need for alternative methods is clear and biosensors have become in recent years a feasible alternative to animal sacrifice. Vilariño *et al*. [153] reviewed the use of biosensors as alternative screening methods for toxins with particular focus on the SPR technology. They concluded that for most groups of toxins there are specific biosensor technologies available, with enough sensitivity to comply with the regulations. For instance, Marchesini *et al*. [154] described the potential for coupling the SPR-based screening for several paralytic shellfish poisons to mass spectrometry for identification using an antibody-based recovery chip. However, none of these methods has been validated and/or accepted as an alternative to the mouse bioassay. Actually, in most cases these techniques could be used at least as screening methods in order to reduce the number of animal bioassays.

When the immunosensors are applied for toxins detection, it is important to keep in mind that the ability of the antibodies to detect the different members of a toxin group is based on the immune response of a host to the antigen and is not related to the toxic potency of these compounds. Functional and biological receptor-based assays/sensors usually provide a better evaluation of sample toxicity, since the measurement is based on the mechanism of action of the toxin. However, the robustness and portability of these functional/receptor-based techniques is not as good as that of immunosensors, because receptors and cells are usually less stable than antibodies.

3.4 Antibiotics

Veterinary drugs are used to treat disease and improve health in animals, analogous to pharmaceuticals in human beings. However, the potential of provoking development of antibiotic-resistant bacteria by the widespread use of agricultural antibiotics has stimulated intense debate. This antibiotic resistance may spread to other microbial populations causing resistance to standard anti-microbial treatments and thus presents a threat to human and animal health [3]. The seriousness of the antimicrobial resistance issue led to an EU-wide ban on the use of antibiotics as growth promoters in animal feed in 2006. Today, antibiotics are allowed to be added to animal feed only as veterinary medication. However, this EU regulation did not result in a decrease in the use of veterinary medicines and, due to their massive use, unwanted residues may still be found in food products.

For the bioreceptor-based detection of antibiotics in food and related products (e.g. blood, urine, renal pelvis fluid, etc.) microbial inhibition screening test and immuno- or receptor-based screening assays are the two mainly applied techniques. Because of their high cost-effectiveness and broad spectrum characteristics, microbial inhibition methods are preferred for large scale monitoring programs on veterinary drug residues. These methods rely on growth inhibition of a susceptible bacterium in the presence of the antibiotic compound. Many test systems have been developed based on this principle, and methods using one to seven agar plates have been reported. The fast antimicrobial screening test (FAST) is a one-plate microbial method with *Bacillus megaterium* that requires a minimum of 6 h for development [155]. The Premi® test uses a vial containing spores of the thermophile *B. stearothermophilis* in agar, and acts similarly to a single-plate method [156]. This test is more rapid, with development times typically from 3 to 4 h. A similar test, the kidney inhibition swab (KIS™) test of Charm Sciences uses a differently configured vial of *B. stearothermophilis* spores in agar, and also allows results within 3-4 h. These three fast tests were compared for screening antibiotic residues in beef kidney juice and serum [157] and one of the conclusions was that there is not one rapid screening microbial inhibition assay for antibiotics that is ideal for all analytes. To cover all possibilities, one would have to run a number of different assays or separate plates. Such a five-plates test has been described [158], and comprises various microbes for the group-specific identification of antimicrobial residues in slaughter animals, the so-called Nouws antibiotic test (NAT screening). The NAT screening combines a simple and efficient sampling and sample processing strategy with a high detection capability because it detects the great majority of antibiotics used in veterinary medicine at or below their

maximum residue levels in kidney. However, it requires a rather lengthy incubation time of 16-18 h.

A faster alternative for the microbial inhibition assay is a whole-cell-based bioassay, also named whole-cell biosensor, which has been described for the detection of tetracyclines [159]. This assay is based on a genetically engineered luminescent bacterial strain that contains the regulation unit of tetracycline resistance factor (tetracycline-responsive element) to control the expression of the luciferase operon. This results in a tetracycline-dependent light production. The time needed for optimal induction of light emission was 90 min. The sensor cell allowed freeze-drying without any loss of sensitivity or overall performance which simplifies the applicability of the assay system. This tetracycline group-specific bioassay was further modified to meet the EU MRL for tetracycline residues in poultry tissue (100 ng g^{-1}) by adding membrane-permeabilizing and chelating agents and sensitivities of 5 ng g^{-1} for doxycycline, 7.5 ng g^{-1} for chlortetracycline and 25 ng g^{-1} of tetracycline were reached [160]. The assay is performed in a 96-well microtiter plate format, allowing simultaneous analysis of several samples within 4 hours and with little preparation. Whole-cell biosensors have the potential to displace growth inhibition assays as the favored method for tetracycline residue screening, since they are better suited for high-throughput analysis and achieve similar sensitivities. However, such bioassays have not been described yet for other kinds of antibiotics. The use of the multidrug-binding repressor protein (QacR) from *S. aureus* [161] might offer an interesting approach for the future development of a multi-drug biosensor. Currently, whole cell biosensors incorporating various microbial reporters are widely used in pharmaceutical drug discovery [162] and for monitoring environmental chemical contaminants [163]. Their application range will most likely expend in the near future to include the detection of antibiotics in food as well.

The most frequently used immunochemical method for antibiotics detection is the ELISA in the 96-wells microtiter plate format. These days, many ELISA kits to detect specific antibiotic compounds are commercially available. In general, they are sensitive and easy to use, have a high specificity, require minimal sample preparations, and are therefore suitable for the screening of a large number of samples in a short time (about 2-3 h). These tests can be used within food-producing facilities. For the detection of groups of compounds, group-specific antibodies were described for fluoroquinolones [164], sulfonamides [165, 166] and benzodiazepines [14] in which generic structures were used for the development of antibodies. Penicillin-binding protein was also used for the detection of

the antibiotics from the beta-lactam group in different food matrices[167]. It was immobilized to a microplate and the amount of a bifunctional reagent (with ampicillin and digoxigenin as functional groups), measured with anti-digoxigenin conjugated with horseradish peroxidase, was used to quantify the amount of beta-lactams present in the sample extracts. A multianalyte screening ELISA for sulfonamides, fluoroquinolones and beta-lactam antibiotics in milk, using three class-selective bioreceptors in a planar microarray configuration, was also recently described [10]. LFDs are much faster antibody-based assays, which can be performed in minutes. In these tests, all ingredients are already present in the test device, and the sample (extract) is needed only to perform an assay. Therefore, this format is ideal for some food-producing facilities and field applications. Such fast tests are described for the detection of sulfadimidine in calf serum [168], sulfonamides in eggs and chicken muscles [169], (dihydro) streptomycine [170] and cephems [171] in milk. Based on this technology, Unisensor (Angleur, Belgium) developed a receptor-based assay dipstick format (Twin sensor^{BT}) for the rapid detection of β-lactams and tetracyclines molecules in raw milk. Other examples of commercially available products are the Rapid One Step Assay (ROSA®) tests for β-lactams, tetracyclines, enrofloxacin and sulfadimethoxine/sulfamethazine of Charm Sciences Inc. (Lawrence, MA, USA). The commercial availability of these rapid qualitative tests is still limited to a few antibiotics.

Besides ELISAs in the traditional 96 well plate format and LFDs, bioassays have been applied for antibiotics detection using newly emerging technological platforms. A more recent approach to screen animal products for veterinary drugs consists of the application of biomolecule-based biosensors. While bioassays or cellular biosensors utilize the response of whole cells to detect biologically active agents, these biosensor instruments use a biological recognition element (e.g. antibodies, enzymes, lectins, receptors and nucleic acids) in close contact with a signal transduction element (e.g. optical, acoustic, and electrochemical) connected to data acquisition and processing systems [163]. Thus, the signal from the biological element is converted to a quantifiable signal, e.g., electrical. Enzymatic biosensors utilize specific enzymes for the capture and catalytic generation of the product, which is then directly determined using different transducers (e.g. electrochemical, optical, photothermal, amperometric, and acoustic). In contaminant analysis, enzyme biosensors have been largely used for organophosphorus and carbamate pesticide and herbicide analysis, with fewer applications being reported for antibiotics, e.g. for the detection of penicillins using penicillinase [172]. Antibody-based biosensors (immunosensors) are frequently described with transduction elements based on

piezoelectric, electrochemical, and optical components. For the detection of antibiotics, electrochemical and optical immunosensors are most frequently applied. Electrochemical biosensors include potentiometric and amperometric immunosensors. The potentiometric immunosensors are based on the change in potential that occurs when an antigen in a sample reacts with the corresponding antibody previously immobilized to an electrode. The potential difference between an antibody-immobilized electrode and a reference electrode is a function of the analyte in the sample. Amperometric immunosensors rely on the measurement of current generated when an electroactive species is either oxidized or reduced at an antibody- (or antigen-) coated electrode to which an analyte (or antibody) binds specifically. Zacco *et al.* [173] developed a novel electrochemical immunosensing strategy for the detection of sulfonamide antibiotics in milk based on magnetic beads coated with class-specific anti-sulfonamide antibodies and a sulfonamide-peroxidase as tracer.

Well established SPR-based optical biosensors, provide commercially available platforms for several food related compounds. Haughey and Baxter [174] published an overview of the Biacore Q kit-based assays for veterinary drug tests in foodstuffs which included tests for antibiotics, β-agonists, and antiparasitic drugs. A disadvantage of Biacore Q is that only one of the four available FCs can be used at the same time. In the Biacore 3000, the four FCs can be serially connected and simultaneously detected. With such a biosensor, direct biosensor immunoassays (BIAs), using monoclonal antibody-coated biosensor chips, were developed which detected gentamicin [175] and (dihydro)streptomycin [176] in milk far below the maximum residue limits (MRLs). However, with milk, interferences were observed which were probably due to the nonspecific binding of milk proteins to the protein-coated sensor chips. Better results, less matrix interferences and more stable chips, were obtained in the inhibition BIA in which four aminoglycosides (gentamicin, neomycin, kanamycin and a streptomycin derivative) were directly immobilized (without coupling to proteins) onto the sensor surface in the four flow channels of the Biacore 3000 that were serially connected [37]. These flow channels were used in combination with a mixture of four specific antibodies. Milk samples were diluted ten times in the antibodies-containing buffer and the limits of detection (LODs) in milk were far below the MRLs (varying from 100 to 1500 ng mL^{-1}) and the total run time between samples was 7 min. These four-channel Biacore systems are expensive and have limited multiplexing possibilities. Moreover, the antibodies are too specific for the simultaneous detection of antibiotics from different groups and the

systems are therefore less suitable for control agencies and food industries to provide an increased and more efficient control on food contaminants (such as antibiotics) in the food chain. Cheaper alternatives and more extended multiplex systems are needed. The application of a low-cost SPR-based prototype biosensor system (Spreeta™) has been described previously [177] in which the sensitivities with inhibition assays for endocrine disruptors were comparable to those obtained with a Biacore 3000. However, this system was less robust and equipped with a single flow-channel only. Alternative eight-channel SPR sensor instruments were developed and used for the detection of low molecular weight endocrine-disrupting compounds [178] and an environmental contaminant in a miniaturized and portable format [41], which are interesting approaches for future research. The SPR imaging (iSPR) technology takes multiplex SPR analysis a step further. Rebe Raz *et al.*[42] used the IBIS iSPR for the simultaneous detection of seven antibiotics in milk. By multiplexing seven immunoassays in a competitive format, they were able to measure all the target compounds at parts per billion (ppb) levels in diluted skimmed milk, within 10 minutes.

Another interesting automated CL-microarray technology (Evidence®) is marketed by Randox (www.randox.com) in which 25 immunoassays can be performed simultaneously. They supply arrays for growth-promoters and antimicrobials but a major disadvantage is that it is a closed system which is not suitable for assay development. Next to these commercially available systems, Chen *et al.* [179] developed a simple and practical biochip system with drug-protein conjugates array spotted onto activated agarose surface-modified glass slides. They employed fluorescently labeled antibodies for the simultaneous detection of eight antibiotics in six sample extracts using a laser confocal scanner. Knecht *et al.* [180] employed an indirect competitive ELISA format to develop an automated parallel affinity sensor array (PASA) for the rapid analysis of ten antibiotics in milk. Microscope glass slides were used for the preparation of microarrays of hapten-protein conjugates which were processed in a flow cell. A mixture of ten monoclonal antibodies in combination with an enzyme-labeled secondary antibody was used for the CL signal detection with a sensitive CCD camera. All liquid handling and sample processing was fully automated, providing analysis of a milk sample within less than 5 minutes.. This PASA system proved to be the first immunochemical biosensor platform having the potential to test for numerous antibiotics in parallel.

The suspension array technology in the flow cytometer (Luminex) was applied for the detection of sulfonamides in milk [181] and in blood serum, meat drip and eggs [182] using

recombinant antibody and for the simultaneous detection of aminoglycosides and sulfonamides in milk and blood serum [183]. Liu *et al.* [184] used the same technology for the simultaneous detection of chloramphenicol, clenbuterol and 17-beta-estradiol. According to them, this technology presented a high-throughput combined with simple operation, high sensitivity and at low cost.

Although QDs have been proven to be suitable labels in bioanalysis, their application in quantitative immunoassays is still limited. Ding *et al.*[185] developed a competitive fluorescence-linked immunosorbent assay (cFLISA) in a microtiter plate for the detection of sulfamethazine in chicken muscle tissue extracts using a commercially available QD (QD 655 (Quantum Dot Corp, Hayward, CA, USA) as the fluorescent label coupled to the secondary antibody. The same QD was used for the detection of enrofloxacin in chicken muscle tissue [186] and the high emission amplitude of the QD (655 nm) led to significant improvements in the signal to noise ratios of the final detected signals. Peng *et al.* [72] described the simultaneous determination of five chemical drug residues (dexamethason, gentamicin, clonazepam, medroxyprogesterone acetate and ceftiofur) in one well of a microplate using a mixture of five antibody-coated cadmium telluride (CdTe) quantum dots in an indirect competition fluorescent-linked immunosorbent assay (ic-FLISA). They described this technology as being less time-consuming than the ELISAs and sufficiently flexible to be used in other systems for the simultaneous multicolor detection of drugs.

3.5 Environmental Contaminants

Environmental security is one of the fundamental requirements of our well being but is a major global challenge. Environmental contaminants are chemicals that accidentally or deliberately enter the environment, often, but not always, as a result of human activities. Some of these contaminants may have been manufactured for industrial use and because they are very stable, they do not break down easily and may enter the food chain. Other environmental contaminants are naturally-occurring chemicals, but industrial activity may increase their mobility or increase the amount available to circulate in the environment, allowing them to enter the food chain at higher levels than would otherwise occur. A wide variety of environmental contaminants is released to the environment every day from residential, commercial and industrial sources and have been detected in foods. Many of these releases, also referred to as discharges, may not pose a threat to the public and the environment. However, a significant release of a contaminant/hazardous substance has the potential to impact human health or the

environment. These range from metals and "ionic" species like perchlorate to organic (carbon-based) substances, including the so-called "persistent organic pollutants" or POPs (named for their ability to exist in the environment for prolonged periods without breaking down). Legacy POPs such as polychlorinated biphenyls (PCBs) have been banned for industrial or agricultural use for many years, but remain in the food chain. Other more recently identified POPs, such as brominated flame retardants, have been found in the environment and the food chain. The persistence in the environment of many organic chemicals like PCBs, polycyclic aromatic hydrocarbons (PAHs) and many others is of great concern because these contaminants may be accumulated through the food chain resulting in higher concentrations in humans and animals. PCBs have been associated with immunological abnormalities, reproductive dysfunction, and liver and thyroid disorders. They also interfere with the endogenous hormone systems and are referred to as endocrine-disrupting chemicals (EDCs).The evidence for damage to human health and wildlife from low level pesticide exposure is also increasing [48]. The lack of monitoring and information on agrochemicals may indirectly help their release into the environment via enormous numbers of urban, agricultural and industrial processes. Because some of these products can endanger ecosystems and persist for long periods in soils and water resources, environmental-related legislation has focused on the hazard assessment of pesticides to control water quality. Current toxicity risk assessments are based on single substance, whilst people and ecosystems are generally exposed to very complex mixtures. Therefore, it is no longer sufficient to detect one analyte per sample and the evaluation of several compounds at the same time is required. Next to these POPs and pesticides, pharmaceuticals and their metabolites can reach water through sewage systems, industrial discharges, effluents from sewage treatment plants, aquaculture, and livestock farming and should be considered as priority water contaminants [187]. They include a hundred substances which are very different as regards chemical-physical properties and environmental behavior and can reach water concentrations of ng L^{-1} to µg L^{-1} and some are considered ubiquitous. In addition to reducing and/or eliminating the amounts of contaminants into the environment, there is a need to develop techniques that can detect and monitor these pollutants in a sensitive and selective manner to enable effective remediation [188]. Due to their integrated nature, biosensors are ideal for environmental monitoring and detection as they can be portable and provide selective and sensitive rapid responses in real time. In their reviews, Farré *et al.* [189], Jiang *et al.* [190] and Suri *et al.* [13] described the great potential of several immunosensors for the rapid detection

of pesticide residues in food and the environment. However, most of them were single-analyte detectors which is a major disadvantage concerning the possible presence of many pesticides. Additional challenge is the development of different types of antibodies that could be used for class-specific monitoring of pesticides and the development of multi-analyte detection systems[191]. Mauriz *et al.* [48] described a multi-analyte SPR immunoassays for environmental biosensing of the pesticides DDT, chlorpyrifos and carbaryl in a two-channelled biosensor (β-SPR of SENSIA S.L., Spain) with sensitivities ranging between 18 and 50 ng L^{-1}. Nichkova *et al.* [192] described the application of two commercially available QDs as labels in an immunoassay microarray for the simultaneous microscopic detection of two biomarkers of exposure to two major classes of compounds: pyrethroid insecticides and triazine herbicides. Guo *et al.* [193] developed a lateral-flow strip test for the simultaneous detection of carbofuran and triazophos using two specific gold-labeled monoclonal antibodies as detector reagents with detection limits in spiked water at 32 and 4 μg L^{-1}, respectively. Another immunosensor with higher multiplexicity was described as the European RIver ANAlyzer (RIANA) which is based on total internal reflection fluorescence (TIRF) by which several analytes (such as atrazine, bisphenol A, and estrone) were detected simultaneously in water at or below the part per trillion level [194]. The experiences gained with this system were utilized to expand the multi-analyte analysis capability for the simultaneous measurements of up to 30 analytes. This research resulted in an automated water analyzer computer-supported system (AWACSS) that can measure several organic pollutants at the low ppt level in a single analysis within few minutes and without any prior sample pre-treatment steps [195, 196]. Weller *et al.* [16, 197] described the application of the parallel affinity sensor array (PASA), based on CL-read out, for environmental contaminants in water. Reagents like antibodies or haptens were immobilized on a glass slide forming a biochip with an active area of about 1.8 cm^2 with a spot density of up to 9 spots per mm^2, which corresponds with an array of 1600 spots. However, they only demonstrated this miniaturized sensor with a few analytes (trinitrotoluene (TNT), 2,4-D and triazines (atrazine and terbuthylazine)) for which the lowest detection limit (20 ng L^{-1} in water) was obtained with terbuthylazine [16].

Immunoassays are very specific, hence for a broader detection of compounds or bio-effect related detections, other biomolecules (e.g. enzymes, receptors or transport proteins) or whole cells are applied.

Enzymes were among the first recognition elements to be incorporated into biosensors. Enzyme biosensors are prepared by attaching to the electrode surface an

enzyme whose products can be measured after the degradation of a substrate. Such systems usually involve the catalysis of redox reactions where either the substrate or the product is electrically charged [198]. Environmental pollutants like parathion, nitrate, and formaldehyde can be detected by sulfite parathion hydrolase, nitrate reductase, and formaldehyde dehydrogenase. Additionally, several biosensors for pesticides and toxic metals monitoring are based on the inhibition of enzymes. Organophosphate hydrolase (OPH) and acetylcholinesterase (AChE) are the two enzymes that have been widely applied in enzyme biosensors for the broad specificity organophophate detection [188] and they operate by inhibition of the enzyme activity (AChE) or as substrates (OPH). Enzyme-based biosensors have also been used for the detection of phenolic estrogens (e.g. phenol, catechol, bisphenol A, genistein, quercetin, nonylphenol, and diethylstilbestrol) using the ability of tyrosinase to catalyze the oxidation of the phenolic estrogens to *o*-diphenol and *o*-quinone [188]. Enzyme biosensors for detecting metal ions rely either on enzyme inhibition or activation methods. In enzyme inhibition methods, metal ions normally combine with thiol groups present in the enzyme structures resulting in conformational changes affecting the catalytic activity. Enzymes such as horseradish peroxidase, alkaline phosphatase, oxidases, urease, L-cysteine desulfhydrolase and invertase have been utilized in the detection of various metals such as arsenic, silver, mercury, cadmium, lead, copper and zinc. The lack of selectivity is described as the major disadvantage of this inhibition assays as some enzymes are inhibited by several metals and even some anions and pesticides. Metal determination by enzyme activation is described as much more selective because fewer metal ions can activate a particular enzyme.

Receptors are supramolecules on the surface of a cell or inside it, that selectively bind specific substances and can be used as biological recognition elements in biosensors. For example Habauzit *et al*. [199] used a SPR sensor for the determination of estrogenic compounds in water using the ER dimerization properties. Estrogenic compounds, such as 17β-estradiol, estriol, estrone, ethynyl estradiol, activated the dimerization process at different concentration levels. They demonstrated the direct detection of 17β-estradiol at concentrations above 1.4 μg L^{-1} and concluded that this method could be a good way to measure the estrogenic potency of compounds and their presence in water. Such an SPR biosensor was also used for the detection of chemicals which may interfere with the thyroid system [200]. There, inhibition assays with the two main thyroid hormone transport proteins, T4 binding globulin (TBG) and transthyretin (TTR), were used in combination with a T4-coated biosensor chip and the most potent binding was observed with

hydroxylated metabolites of the brominated diphenyl ethers (BDEs). Whole-cell biosensors are one of the newest tools used in environmental monitoring [201-203]. Such biosensors are based on genetically engineered microorganisms or cells in which reporter genes are fused to responsive promoters. Currently, a variety of environmental pollutants (e.g. PAHs, PCBs and dioxins), can be detected with these biosensors. Bovee *et a*l. [204] used the mammalian cell-based CALUX (Chemical-Activated LUciferase gene eXpression) bioassay for the determination of dioxins and related compounds in bovine milk. An equivalent of 67 mg fat was tested per experimental unit, resulting in a low limit of quantification of around 1 pg. TEQ (Toxic EQuivalent) per g fat, which is suitable for the screening of dioxins and dioxin-like PCBs. Within our institute, the CALUX bioassay, was accredited for food and feed analysis. A recombinant yeast cell-based estrogen bioassay, expressing human estrogen receptor α and yeast enhanced green fluorescent protein in response to estrogens, was developed [205] and applied for the screening of estrogenic activity in calf urine [206] and animal feed [207]. The cell-based biosassays provide an insight on contaminants toxicity and bioavailability and thus are in particularly useful for the detection of unknown agents.

4 Concluding Remarks

Multi-analyte bioassays provide cost-efficient and rapid analytical solutions and thus contribute to continuous improvement of life quality in both industrialized and developing countries. The outlook, provided in this review suggests that the need in multiplexed analysis in food safety and environmental is met by rapidly developing bioassay–based technologies (Figure 2.4). The described multi-analyte methods feature versatile innovative technological platforms and implement a range of biorecognition elements. The choice of the multi-analyte bioassay is essentially dictated by the particular application in mind. Not only the target analyte is the decision driving force, but also the environment where the analysis need to be performed and the implications of the obtained results. For instance when milk samples need to be tested for antibiotic residues prior entering the manufacturing line rapid and easy-to perform method is desired with semi-quantitative capabilities. However, when monitoring food and environmental contamination is performed by regulating authorities, more accurate and high-throughput screening method is needed. Additionally, the level of the desired multiplicity influences the choice of the biorecognition factors employed in the bioassay. When a wide target screening range

is needed (for instance when screening for environmental contaminants) generic antibodies, receptors or enzymes are preferred.

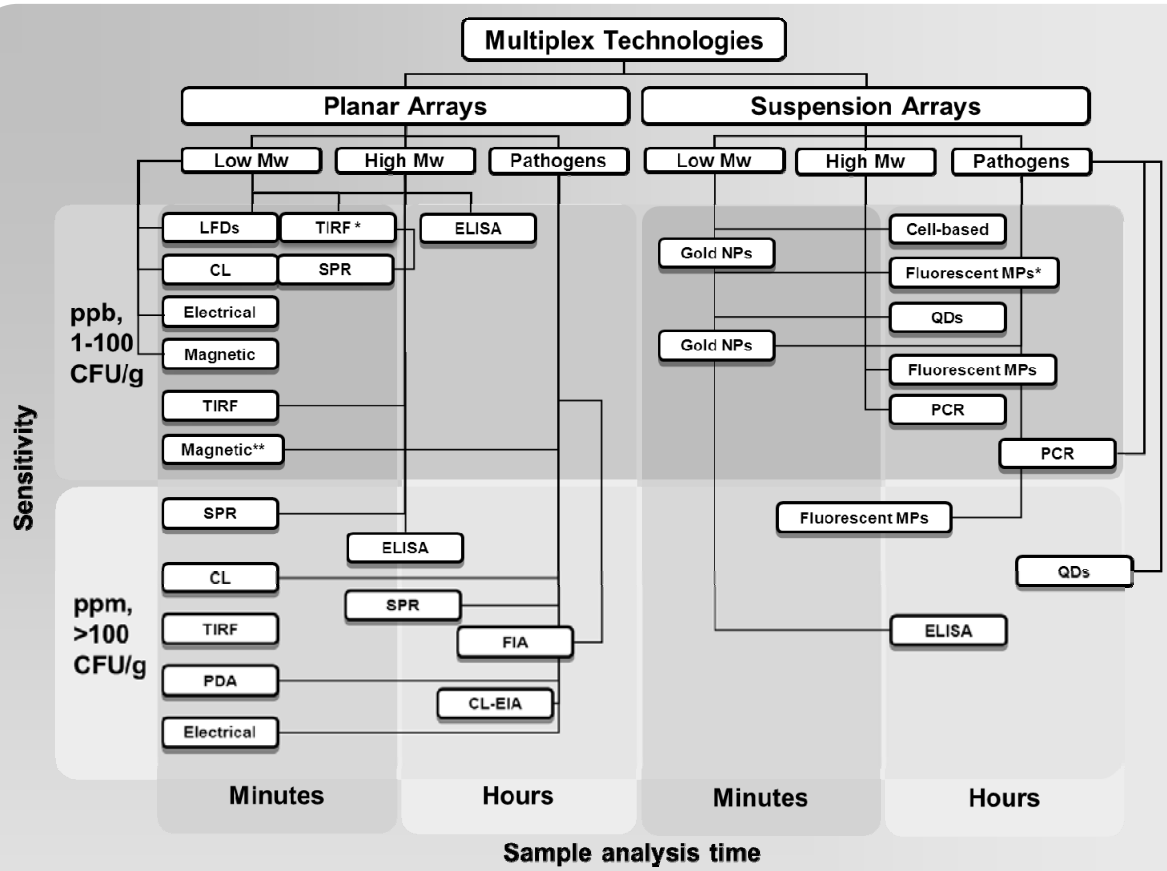

Figure 2.4 An overview of bioassay-based multiplexed technologies currently applied for food and environmental analysis. Multiplexed technologies are distinguished first accordingly to the array platform (planar or suspension), and classified according to application (low/ high molecular weight analytes and pathogens detection). Then, they are classified with regard to sensitivity (parts per billion (ppb)and parts per million (ppm) for low and high molecular weight analytes , and colony forming units for pathogens ; in gram food or ml buffer (depending on the assay)) and with regard to assay time (minutes or hours). Abbreviations used include: CL-chemiluminescence, TIRF-Total internal reflection fluorescence, ELISA-enzyme linked immunosorbent assay., LFDs- lateral flow devices, SPR-surface plasmon resonance, PDA-polydiacetylene –based sensor chip , FIA- Fluorescence-based immunoassay, CL-EIA – chemiluminescence -based enzyme linked immunoassay, NPs- nanoparticles, MPs-microparticles, QDs-quantom dots, PCR-polymerase chain reaction. *- parts per trillion sensitivity. ** pM sensitivity of PCR products.

The majority of the literature describes development of the multi-analyte methods, presenting their potential application and demonstrating proof of concept in food or environmentally relevant samples. Very few novel technological platforms have been thoroughly studied, validated and even less commercialized. This might be the reason for still limited dissemination and application of these technologies to routine analysis. This review also suggests that the field is currently dominated by multiplexed technologies based on planar arrays, mostly biosensors. Most likely due

to inherent benefits of biosensors, such as, short measurement times and automation. Among the biosensor-based multiplexed assays, the NRL array biosensor and the PASA analytical microarray systems are the closest to being widely applied for food and environmental monitoring. Both systems have been used to detect a variety of analytes in different food matrices and both demonstrated short measurement times, high sensitivity and high multiplexing capabilities. Moreover, they have been automated and reduced in size, and thus reached the full potential of the biosensing system. For most environmental and food contaminants, the trend of developing multiplexed bioassays is towards increasing throughput and automation along side with reducing the costs of the analysis. These are the main bottlenecks of the traditional methods and need to be efficiently overcome by the novel multiplex approaches. Traditional methods are still needed for confirmation purposes; however initial screening of suspected samples will be dominated by the multiplexed systems, described here, already in the near future. Outside the analytical laboratory, food and environmental monitoring will greatly benefit from the development of portable and self sustainable biosensors.

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Chapter 3

Imaging Surface Plasmon Resonance-based Biosensing

Imaging Surface Plasmon Resonance (iSPR) biosensing is based on monitoring molecular interactions with SPR in a spatially resolved manner. This approach allows label free and real-time measurements of binding events which occur on the microarray surface. iSPR has been applied throughout this thesis as an analytical method for multiplexed detection of hazardous compounds *in food. Here, the basic principals of the iSPR technology are described, with a special emphasis on the aspects that are important for bioassay development.*

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Surface Plasmon Resonance Phenomenon

Surface Plasmon Resonance (SPR) was discovered over 100 years ago, when Wood noticed narrow dark bands in the spectrum of diffracted light from a grated metal surface [1]. Theoretical analysis of the phenomena, 40 years later, led to a conclusion that the observed anomality in light diffraction is related to surface plasma waves (SPW). SPW, or surface plasmon polaritons, are waves propagating along a conducting surface. When metal surfaces are illuminated, free electrons can oscillate in resonance with the light, trapping it on the surface. The resonant interaction between the surface electrons oscillation and the electromagnetic field of the light produces the SPW and results in two phenomena as follows [2-5] (Figure 3.1). First, the field component perpendicular to the surface, called evanescent wave, is enhanced near the surface decaying exponentially with increasing distance from it. The decay length in the dielectric field above the metal surface is of the order of half light wavelength. Second, the surface plasmons momentum increases, producing a mismatch between photons and surface plasmons at the same frequency. Due to this mismatch, the light momentum needs to be enhanced in order to generate SPW. The missing momentum can be provided by several techniques: prism coupling, scattering from topological defect on the surface (e.g. subwavelength hole) or by systematic corrugation of the surface [5-9]. Kretschmann geometry is the most commonly applied configuration in SPR instrumentation (Figure 3.2). It is a prism coupling technique, where total reflection of light is used for excitation of SPW. When light travels through an optically dense medium, such as glass, to a less optically dense medium, such as buffer, it is reflected at the interface and total internal reflection (TIR) takes place. TIR occurs under the condition that the light angle of incidence is greater than the critical angle required for the pair of optical media. At a defined angle of incident light, the light energy is transferred to the metal film and surface plasmon resonance occurs, causing a reduction in the reflected light intensity. The light angle, at which SPR occurs, depends on the refractive index of the optically low density medium, usually a buffer solution. Monitoring SPR angle or light reflectivity allows quantitation of the refractive index of the solution at the metal film surface [10].

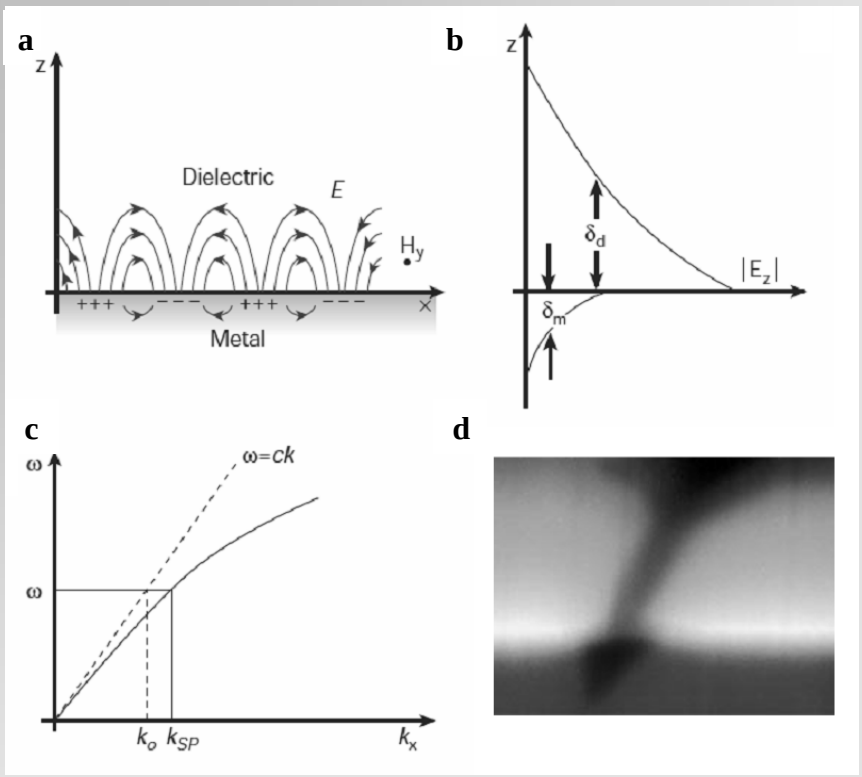

Figure 3.1 Surface Plasmon Resonance phenomenon. **(a)** Surface plasmons (SPs) at the interface between a metal and a dielectric material have a combined electromagnetic wave and surface charge character. They are transverse magnetic in character (H is in the y direction), and the generation of surface charge requires an electric field normal to the surface. This combined character also leads to the field component perpendicular to the surface being enhanced near the surface- the evanescent wave (EW). **(b)** The decay length of the EW in the dielectric medium (δd) above and in the metal (δm). In the dielectric medium above the metal, typically air or glass, the decay length of the field, is of the order of half the wavelength of light involved, whereas the decay length into the metal, is determined by the skin depth. **(c)** The dispersion curve for a SP mode shows the momentum mismatch problem that must be overcome in order to couple light and SP modes together, with the SP mode always lying beyond the light line, that is, it has greater momentum ($\hbar k_{SP}$) than a free space photon ($\hbar k_0$) of the same frequency ϖ. k_0=w/c is the space free wave-vector. k_{SP} is frequency-dependent SP wave-vector. **(d)** Image of the SP photonic bandgap. The SP dispersion curve for a flat surface (here shown as inverse wavelength versus angle) was directly imaged using a modified prism coupling technique. The dark regions correspond to coupling of incident light to the SP mode, and the colors (here shades of grey) are produced on a photographic film by the wavelength of the light used. Adopted with modification from Barnes W. (2003) [11].

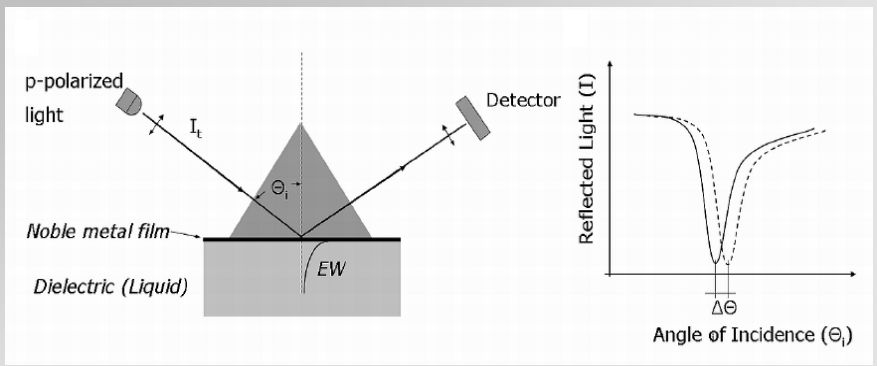

Figure 3.2 Kretschmann geometry set up for prism-coupled excitation of SPW. The wavevector of plane polarized light is enhanced by passing through an optically dense medium (prism, often made of BK7 glass). The coupling between the light wave in the prism and an SPW at the metal is established by the total internal reflection method. The light is totally reflected at the prism base, generating an evanescent wave (EW) penetrating the metal and the extending in to the low–refractive index dielectric medium. The coupling of the SPW to EW is controlled by the angle of the incident light (Θ). When EW is coupled to SPW, the intensity of the reflected light (I) is reduced. Change in refractive index of the dielectric medium close to the metal surface will produce a shift (ΔΘ) in angle of the incident light at which SPR occurs. Adopted with modifications from Homola J. (2002) and Marchesini G. (2008) 4, 12.

Surface Plasmon Resonance-based Sensors

SPR-based biosensors exploit SPR phenomenon to probe molecular interactions. SPR biosensors comprise an optical unit, a fluidic system and a sensor chip modified with a biological element. Fluidic systems are usually a flow-cell or a cuvette which deliver and confine the sample to the sensing surface. The biological element, immobilized on the sensor chip surface, is called ligand and it interacts with analyte molecules in the sample. The optical unit excites and interrogates SPW during the interaction between the ligand and the analyte on the sensor chip surface. When the refractive index on the sensor surface changes, as a result of interaction, it produces a change in reflected light properties. Depending on the light property measured, SPR instrumentation can be classified into angular, wavelength, intensity, phase and polarization modulators [4]. In principal, any biomolecular interaction that produces a change in the refractive index at the sensor surface can be measured. The sensitivity of the SPR biosensor is defined by the sensitivity to refractive index changes and by the binding properties of the biological element which is coating the sensor surface. Both, optical and biological sensitivities, determine the limit of detection and the resolution of the SPR biosensor by influencing the accuracy with which the sensor's output is determined. Thus, the final outcome of the SPR biosensor depends on the interacting pair, the ability of the target analyte to produce a refractive index change and the optical sensitivity of the instrument. The ability to efficiently deliver the target analyte to the surface greatly influences the sensor's output as well, due to the fact that SPR is sensitive only to refractive index changes close to the sensor surface.

The first commercial SPR sensor was based on Kretschman geometry and angular modulation and was launched in 1990 by Biacore, followed by many others [13].The Biacore instrument used in this thesis was the 3000 model (Figure 3.3). This system uses plane-polarized light to illuminate a gold film through a prism in four sensing areas. The angular spectrum of each sensing area is reflected on a linear diode array where the light intensity is measured. The biosensor output is calculated from the shift of the incident light angle at which SPR occurs. This angle shift plotted against time, the so called sensorgram, provides a real time probing of the refractive index change in the SPW-probed range. For example, binding of an antibody in solution to an antigen immobilized on the sensor surface will cause refractive index change and will result in a shift in SPR angle, which will be recorded as a response in the sensorgram. The fluidic system in Biacore 3000

features four serially connected flow-channels (FCs), allowing parallel investigation of 4 binding events. Each FC has an area of 1.2 mm^2, 20 µm height and 0.02 µl volume.

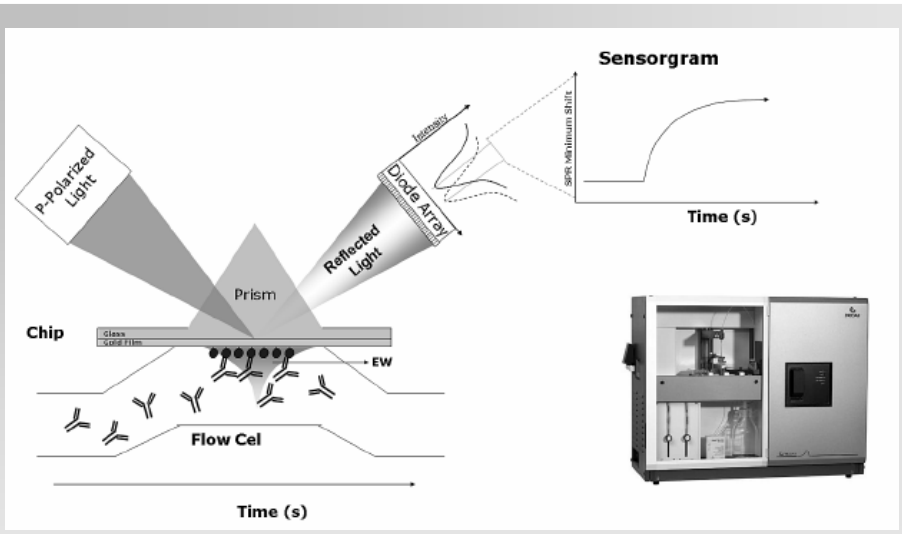

Figure 3.3 Biacore 3000 Surface Plasmon Resonance based sensor. A fan shaped beam of p-polarized light illuminates through a prism the sensor chip surface. The reflected light from the surface is detected by the photodiode array. The minimum of the reflected light (SPR angle) is calculated using an algorithm. On the sensing side of the sensor chip, coated with the ligand, a flow cell is assembled. The sample passes through the flow cell and the analyte interaction with the ligand on the sensor surface is monitored continuously by changes in the SPR angle, producing a sensorgram. Biacore 3000 instrument is equipped with automated liquid handling system, injection port, two syringe pumps, sensor chip docking station and one buffer inlet. Adopted from Marchesini G. (2008) [12, 14].

Recent advances in the SPR instrumentation were mainly focused on providing multi-channel sensing systems, which enable high-throughput and multi-analyte measurements. One of such systems is imaging SPR (iSPR), which measures refractive index changes on the surface in a spatially resolved manner [15-19]. In this thesis IBIS iSPR system was used. It is based on Kretschman geometry and angular modulation of monochromatic (840 nm) plane-polarized light. A 25 mm^2 surface area is illuminated with incident light at different angles which are controlled by a mirror within a range of 8^o (between 66 and 78^o). The angle of the incident light can be adjusted manually with an optical leverage arm. The sensor chip is placed on top of hemispheric prism (BK7 glass) using refractive index matching oil (n=1.518). The images of the illuminated sensor chip surface are captured by a charge-coupled device (CCD) camera. Light reflectivity is determined from the grey values of the pixels and plotted as a function of the scanning angle [20, 21]. Data acquired from the camera are processed by the software and the response is expressed as a shift of the SPR angle in millidegrees. SPR angles are monitored simultaneously on the entire imaged surface using pre-defined regions of interest (ROIs). The relationship between amount of protein adsorbed to the surface and the shift in SPR angle , in IBIS iSPR, is given by 93 m^o per pg mm^{-2} protein [22]. Fluidics on the sensor chip surface is handled by a cuvette or a flow cell. In the flow cell set up, the injected sample is

delivered to the sensor surface and pumped back and forth during the interaction. In the cuvette set up, the mixing is performed by a double needle aspirating and dispensing in close proximity to the sensor surface. The temperature of the sample rack and the flow cell is controlled separately by two Peltier elements. The measurements can be run automatically, accordingly to programmed liquid handling procedures. Raw data are saved to a database file, which is subsequently uploaded to SPR data analysis dedicated software (Sprint). Sprint software allows simultaneous handling of the sensorgrams acquired from all the spots on the sensor surface during sample analysis. Usually data processing involves calibration of the sensorgrams (to eliminate the differences caused by non-uniform surface illumination), zeroing to the baseline before the start of the sample injection and referencing the signal to the blank spot. From the processed SPR data, a response at a certain time can be calculated and exported to Excel. For kinetic analysis, the data have to be exported to Scrubber or similar software.

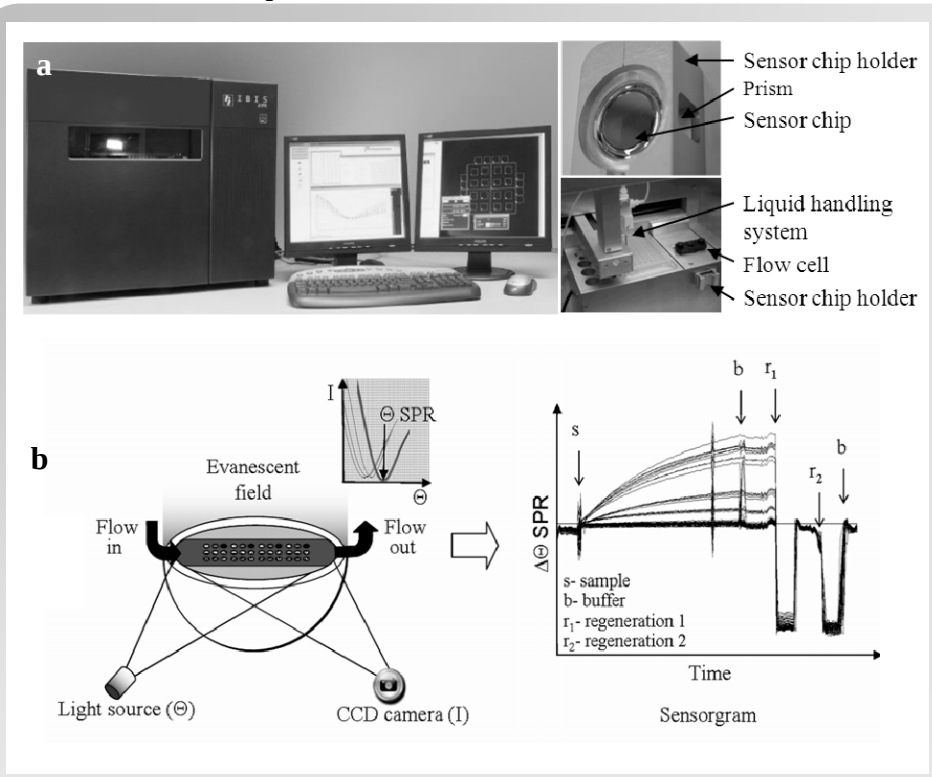

Figure 3.4 IBIS imaging Surface Plasmon Resonan-ce instrument. **(a)** Components of IBIS iSPR instru-ment. IBIS iSPR instrument is equipped with automated liquid handling system, one syringe pump, sensor holder (for sensor chip and prism assembling), one buffer inlet and a flow cell or a cuvette. **(b)** Operational prin-cipals of IBIS iSPR sensor. Sensor chip which was spotted with different ligands is mounted on top of a glass prism and assembled with the flow cell. The surface of the sensor chip is illuminated at different light angles and images of the surface are taken by a CCD camera. For each spot the SPR angle is determined from angle versus intensity plots. The change in SPR angle (sensorgram) is monitored in real time and simultaneously on all the spots during buffer, sample and regeneration solution injections.

Molecular Interactions

Molecular interactions underlie most of the inner and outer-cell processes and play an utmost important part in our understanding of biological mechanisms. SPR biosensors became a widely accepted tool to interrogate these interactions as well as to employ them in bioassays. Depending on the

experimental set-up, SPR data can provide information either on the concentration of the analyte or on kinetic and thermodynamic properties of the ligand-analyte interaction. Due to the fact that SPR offers a possibility to monitor molecular interaction in real-time, kinetic analysis has been a major emphasis of biosensor use. The kinetics of biomolecular binding reaction, in SPR set up, is often described by a simplified 1:1 interaction model between the ligand (B), immobilized on the surface, and analyte (A) in solution, sometimes including correction for mass transport limitation (Equation 3.1) [23].

$$A_{bulk} \underset{k_m}{\overset{k_m}{\rightleftarrows}} A_{surface} + B \underset{k_d}{\overset{k_a}{\rightleftarrows}} AB \qquad \text{(Eq.3.1)}$$

Here k_m is the molecular weight dependent mass transfer rate constant, k_a is the association rate constant and k_d is the dissociation rate constant. The affinity (K) of the binding (or the equilibrium constant) between ligand and analyte is given by the ratio between the association rate constant and the dissociation rate constant. The equilibrium dissociation constant (K_D) is inverse of the affinity constant (K) (Equation 3.2).

$$K = \frac{k_a}{k_d} = \frac{1}{K_D} \quad \text{(Eq. 3.2)}$$

Under mass transport limited conditions, the analyte is first delivered to the sensor surface by means of convection and diffusion and then the binding of the analyte to the ligand takes place. The association rate constant, provides information on how fast the analyte-ligand complex is formed (usually between 1 x 10^{-3} and 1 x 10^{7} $M^{-1}s^{-1}$) and the equilibrium association constant provides information on the association tendency of the complex (usually between 1 x 10^{5} and 1 x 10^{12} M^{-1}). During the association phase in the SPR sensorgram, when the solution containing the analyte is introduced to the sensor chip coated with the ligand, the interaction rate at time, is described by equation 3.3. When the sample injection is finished, and buffer is introduced to the sensor surface, only complex dissociation takes place (Figure 3.5).

$$\frac{d[AB]}{dt} = k_a[A][B] - k_d[AB] \quad \text{(Eq. 3.3)}$$

SPR data, the sensorgram, is mostly fitted with a linear regression model, where the SPR response (R) at a given time (t) is described according to equation 3.4. R is proportional to the amount of the complex formed on the surface and R_{max} is a maximum analyte binding capacity of the surface.

$$R_t = \frac{k_a[A]R_{\max}}{k_a[A]+k_d}(1-e^{-(ka[A]+kd)t}) \quad \text{(Eq.3.4)}$$

Plotting dR/dt versus dR, gives linear transformation of the data, to which linear regression can be applied. The slope of the obtained line is the observed kinetics constant (k_{obs}) and is described as given in equation 3.5, where C is the concentration of the analyte .

$$k_{obs} = k_a \times C + k_d \quad \text{(Eq. 3.5)}$$

The association constant (k_a) is derived from k_{obs} by plotting k_{obs} versus C, the analyte concentration. k_d is usually derived from the dissociation phase, because it is usually very low and can not be reliably derived from $k_{obs.}$ Kinetic analysis of the SPR data is usually done by an evaluation software supplied with the instrument (such as Biaevaluation) or can be exported in to other kinetic analysis dedicated software (such as Scrubber and Clamp). The SPR data, describing molecular interaction, does not always fit the assumed 1:1 interaction model, suggesting more complex binding kinetics. In such cases, methods such as integrated rate equations and numerical integrations are applied [24]. There are also a number of experimental artifacts that can interfere with kinetic analysis when using SPR biosensors. They include, among others, surface heterogeneity, aggregation, avidity and crowding. Using highly pure bioreagents, routine instrument cleaning and maintenance, proper experimental design and data analysis can help to avoid those problems [25].

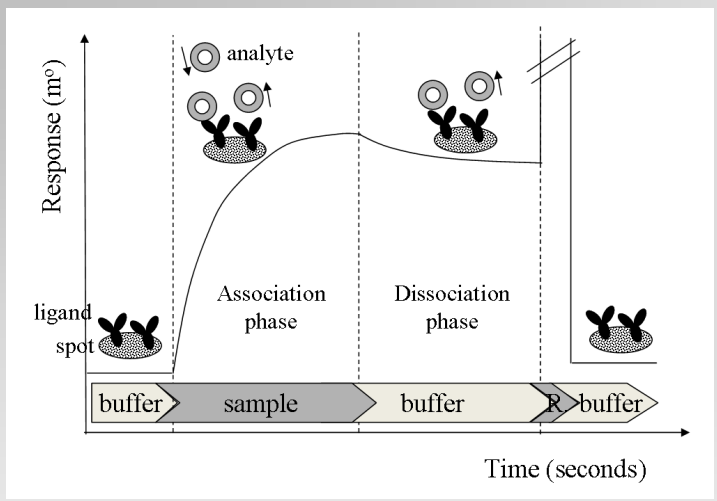

Figure 3.5 Scheme representing a typical Surface Plasmon Resonance sensorgram. Ligand is immobilized on the sensor surface (spot) and the surface is equilibrated with the runing buffer until a stable baseline is obtained. Then, a sample containing analyte is injected and ligand-analyte interaction takes place (association phase, where the ligand-analyte complex is formed). Next, the sample is removed and the surface is flushed with the running buffer (dissociation phase, where ligand-analyte complex falls apart). Finally the surface is regenerated with regeneration solution (R), which contains usually one or a combination of the following : acid, base, organic solvent, detergent, chelator and etc.. This step disrupts the ligand-analyte complex, leaving the sensor chip surface available again for the next measurement. To verify regeneration, an additional equilibration step is performed with the running buffer.

Surface chemistries Sensor surface functionalization with the biological element plays a crucial role in the biosensor development. The quantity and activity of the ligand

on the surface affect the sensor's output and determines its analytical capability. Surface properties also provide the environment where the molecular interaction takes place, and thus influence the binding. The simplest surface coating with ligand is merely by adsorption to the metal film, for instance adsorption of antibodies to a bare gold film [26]. However, when adsorption of proteins to the surface takes place, denaturation might occur and hence loss of activity. Moreover, the bare gold surface may unspecificaly adsorb proteins from the sample, masking the specific ligand-analyte interaction. To produce stable and defined surfaces, which can be used through multiple measurement cycles, covalent attachment of the ligand is usually employed. This can be achieved by using a linker layer between the ligand and the surface. For example, alkanethiols with suitable reactive groups to couple the ligand on one end and gold-complexing thiol group on the other [27]. The thiol molecules will self-assemble on the gold surface, creating a mono-layer of ligand. In 1990s Lofas introduced a revolutionary approach towards ligand immobilization on the surface by using a hydrogel composed of carboxymethyldextran (CM-dextran) [28]. CM-dextran yields a three dimensional matrix for ligand immobilization and offers several advantages. It provides a hydrophilic environment for molecular interaction and maintains the mobility of the immobilized molecules, enabling better thermodynamic and kinetic characterization of the binding event [29]. It improves the sensitivity of the biosensor by increasing the amount of immobilized ligand in the SPW probed field and reduces non-specific binding of the sample components to the sensing surface. Carboxyl groups on the dextran can be easily derivatized for covalent attachment of molecules via amine, carboxyl, sulfhydryl and aldehyde groups (Figure 3.6). Currently many hydrogel based sensor surfaces are in use, including CM-dextran at various lengths, cross-linkage degrees and carboxylic acid substitution degrees, linear polycarboxylate, agarose, alginate , pectin, and more. To characterize interaction of molecules with membrane surfaces, sensor surfaces can be adapted to work with lipophilic molecules, such as lipids, liposomes and whole cell membranes. Self-assembled lipid layers can be generated using thiols, as well as lipid bilayer systems [30]. The commercial availability of these surfaces depends on the SPR instrumentation used. Usually the manufacturers of the SPR equipment offer designated sensor chips with different coatings. In some cases, it is possible to purchase sensor chips from a different manufacturer, and there are companies that produce sensor chips suitable for many SPR systems and specialize in sensor surface chemistry [31].

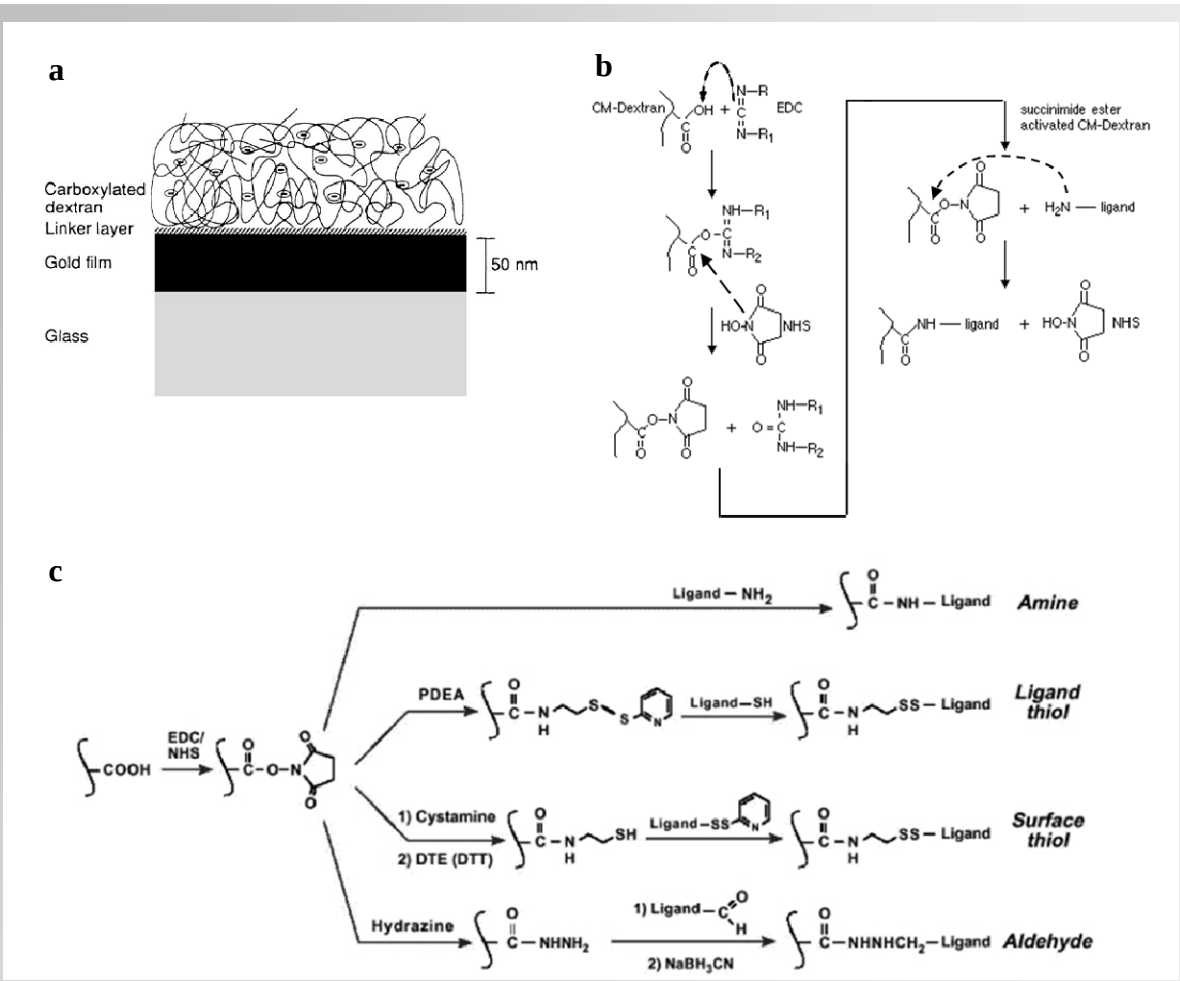

Figure 3.6 Carboxymethyldextran surface and ligand coupling chemistries. **(a)** Schematic representation of a SPR-sensor chip set up. A glass layer is coated with a thin gold film (50 nm), then carboxymethyldextran (CM-dextran) is attached covalently to gold via a self-assembled monolayer of ω-hydroxyalkanethiol. The thickness of the CM-dextran layer can vary anywhere between few nm and 1000 nm. The density of the CM-dextran is controlled by the extent of covalent cross linkage and the charge of the CM-dextran is dependent on the amount of methyl groups substituted with carboxylic acids. In CM5 Biacore chip, most commonly used in SPR biosensing, the CM-dextran is non-cross linked, with a carboxyl substitution degree of 1 carboxyl group per glucose unit and when swollen in physiological solution it extends 100 nm from the surface. Adopted from S. Lofas (1990)[28]. **(b)** Ligand coupling chemistry to CM-dextran mediated by carbodiimide. A Water soluble derivative of carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), is often used for covalent coupling of ligands to CM-dextran via amide bond formation. EDC activates carboxylic group to form an O-urea derivative which reacts readily with nucleophiles, such as amines, to form a peptide bond. Often, *N*-Hydroxysuccinimide (NHS) is used to assist the EDC coupling by forming an intermediate active ester (the product of condensation of the carboxylic group and NHS) that further reacts with the amine function. NHS produces a more stable reactive intermediate which has been shown to give a greater reaction yield. Carbodiimide coupling is favored by a high pH at which the amine groups of the ligand are not charged (pKa $_{Lys[NH2]}$=10.67). However, at high pH most of the proteins are negatively charged and thus are repelled from the negatively charged CM-dextran (pKa $_{[COO-]}$=3.5). Thus coupling reaction of proteins to CM-dextran is usually performed in acidic conditions, and in low salt buffers. The latter prevents protein and CM-dextran charge neutralization by counter ions. Adopted with modification from George A. (2001) [32-34] **(c)** Variations on carbodiimide-mediated ligand coupling chemistry. Succinimide ester can be further modified with 2-(2-pyridinyldithio)ethaneamine hydrochloride (PDEA) to introduce active disulfide group, which will react with thiol groups on the ligand. If the ligand has disulfide groups, the CM-dextran surface can be modified with cystamine and dithiothreitol to create a thiol group on the surface. Thiol coupling approach is often considered to be less efficient due to the fact that the number of available thiol groups for ligand coupling is limited. However, it may be beneficial to preserve ligand activity, in case the ligand has an amine group essential for binding. Thiol coupling also gives a more oriented, and thus more defined immobilization. Other possible coupling chemistries employ aldehyde and carboxyl groups on the ligand. Adopted with modifications from Haasnoot W. (2009) [35].

Biorecognition Elements

In principle, any element with a biological function could be used for biorecognition in a SPR-biosensor: e.g. deoxyribonucleic acid, protein, supramolecule, organelle or a cell. However, to be able to monitor interaction by SPR, the analyte-ligand pair must fulfil two basic requirements. First, one of the binding partners has to be immobilized on the sensor surface. Second, the binding partner in solution has to generate a sufficient refractive index change, within the SPW probed field, upon binding to the ligand on the sensor surface. When these two criteria are met, the bioassay format choice is made. In the direct format, the binding partner is immobilized on the surface and its interaction with the analyte in solution is monitored. The refractive index close to the sensor surface is increased when analyte binds to the ligand, producing a direct relationship between the analyte concentration in solution and refractive index change. In the sandwich format, the binding of analyte to the sensor chip surface is followed by a secondary binding step of an additional molecule, for signal enhancement. In the competitive format, binding of the biorecognition element to the analyte immobilized on the sensor surface is inhibited by free analyte in the solution, producing an inverse relationship between the analyte concentration in solution and refractive index change. Direct and sandwich formats are usually applied for detection of high molecular weight molecules, because these compounds are able to generate a high refractive index changes and they possess multiple binding sites. Low molecular weight compounds are usually detected in a competitive assay. For example, characterization of antibody binding towards its antigenic protein is performed in a direct format and immunodetection of antibiotics is performed in a competitive format [36, 37]. If the SPR sensor sensitivity is sufficient (for instance Biacore 3000) or a compound has high refractive index (for instance aminoglycosides) it is possible to detect low molecular weight compounds in direct format as well [38, 39]. In this thesis, both direct and competitive bioassay formats were used (Figure 3.7). Another consideration point in biosensor assay development is the stability of the ligand. Sensor chips coated with small compounds are generally considered to be more robust, allowing hundreds of measurement cycles. Whereas, in direct format assays, when the biorecognition element is immobilized to the surface, regeneration of the sensor chip might cause loss of activity and restricts utilization of the biosensor. If the measurements are performed in complex samples, the sensitivity of the direct assay format is usually lower than that of the competitive assay format. This is due to the fact that limit of detection (LOD) is calculated in direct assay based on the signal measured in various

blank samples, which matrix components might be slightly different. In competitive format, the signals in each sample are normalized to a signal obtained in the same sample without the analyte, resulting in much lower variability in blanks and thus higher LOD. Eventually, the choice of the biorecognition element and the bioassay format should be considered individually taking into account all the factors described above.

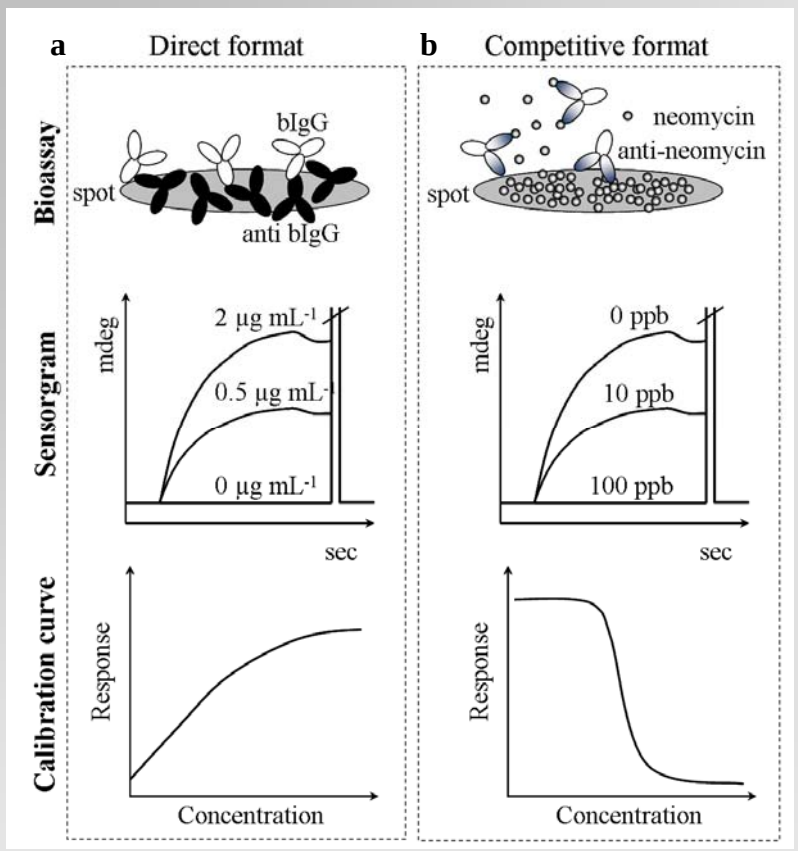

Figure 3.7 Direct and competitive bioassay format used in iSPR-biosensing. **(a)** Direct bioassay format. Here, the binding molecule is immobilized on the surface and its interaction with the analyte in solution is monitored. In this format, there is a direct relationship between the amount of analyte in the sample and the signal acquired with SPR. For instance bovine IgG (bIgG) concentration measurements with anti bIgG on the spot result in a calibration curve which could be fitted with linear (Y= YIntercept + Slope*X) or one-phase association model (Y=Y0 + (Plateau-Y0)*(1-exp(-K*x)), K is the rate constant). **(b)** Competitive bioassay format. Here, the analyte (neomycin for example) is immobilized on the spot. Then the sample is introduced together with the anti-neomycin antibody. The binding of the anti-neomycin antibody to neomycin on the surface is monitored with SPR. There is a competition for antibody binding between neomycin in solution and neomycin on the spot; hence an inverse relationship between the SPR signal and the analyte in the sample is established. The calibration curve in competitive assay format takes a sigmoidal form and can be fitted with non-linear-parameter model (Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC50)), Top and bottom are the plateau values , and IC_{50} is the concentration of agonist that gives a response half way between Bottom and Top .

Application Aspects

Even though SPR biosensors are most often used for kinetic studies of molecular interactions, they have another important application. Label free, rapid and often automated measurements using SPR provide a technique for quantitative detection of analytes [14, 40, 41]. In many cases, SPR-biosensors offer powerful alternatives to already existing methods. For example, in food, they have been successfully used for specific detection and quantification of several molecules, e.g. vitamins, antibiotics, toxins and plant proteins [42-47]. However, the application of SPR biosensors to measurements in “real-life” samples is hindered by negative effect of the

matrix components on the bioassay performance. Nonspecific binding to the sensor chip surface is the most common bottle neck in the SPR-based bioassay development, and sometimes a sample pre-treatment step is needed prior to analysis. Additional drawback of SPR biosensors, so far, has been the low throughput and high costs of dedicated instrumentation. Imaging SPR (iSPR) platforms combine the advantages of SPR sensing with multiplexing possibilities of microarrays [48-54]. There are several commercial iSPR instruments available, e.g. SPRi-Plex™ (Genoptics Bio interactions), ProteOn™ XPR36 (Bio-Rad laboratories), SPRimager®II ARRAY system (GWC Technologies) and IBIS iSPR (IBIS Technologies B.V.). The instruments differ in optics, fluidics, sample handling and sensor surface derivatization. All of these factors greatly influence out-put of the sensor, and the choice of the iSPR instrument is usually made according to the targeted application.

Besides choosing the right SPR equipment, the microarraying method should be carefully considered. Sensor surface preparation for iSPR involves patterning ligands in a microarray manner. Combining optimal conditions for ligands solubility, spot formation on the sensor surface and immobilization efficiency often presents a challenge. For instance, hydrophilic polymers, like CM-dextran, are favourable for SPR studies; however spot formation on such surfaces is difficult. Additionally, when iSPR is applied for concentration measurements, maximal surface load with the ligand is desired, but if a ligand has a high molecular weight, only limited number of molecules fit into the drop that forms the spot on the sensor surface. In such cases, using a spotting technique which utilizes a flow of the ligand on top of the sensor surface has an advantage over drop depositing techniques (Figure 3.8).

From the analytical point of view, iSPR offers many advantages, e.g. label-free and real-time measurements, automation, multi-analyte detection, good sensitivity and absence of sample pre-treatment (Table 3.1). Depending on the compound, sample pre-treatment may be very laborious and time consuming. For instance detection of antibiotic residues in complex matrices such as milk, meat or serum with LC-MS involves multiple sample preparation steps prior to actual analysis. Whereas measuring the same compounds with iSPR , merely demands dilution of the sample in running buffer [55]. For some compounds, such as allergens, the analyte has to be extracted from the sample matrix and thus sample preparation step is necessary for iSPR as well. So far, iSPR platform was mainly utilized for profiling protein-protein and DNA -protein interactions using protein or DNA microarrays, and its analytical potential has not been fully explored yet [56-59]. In

this thesis, the applicability of iSPR platform to quantitative multi-analyte food screening was evaluated.

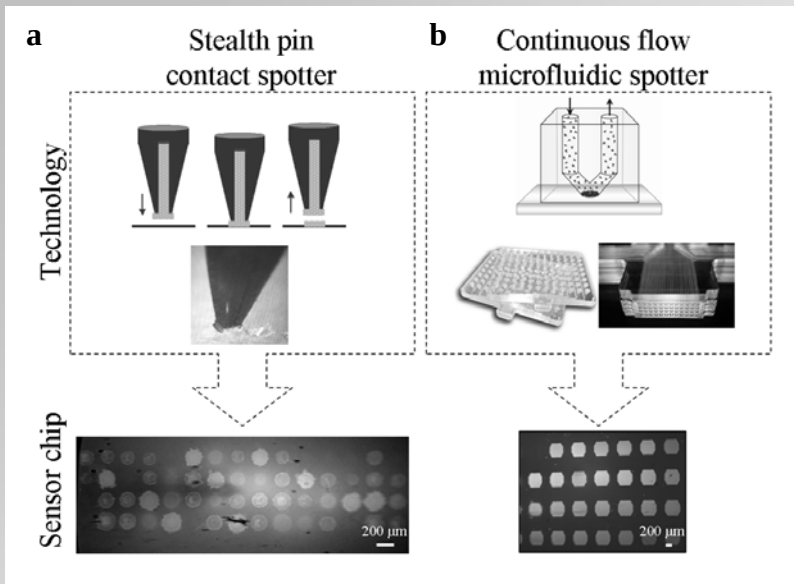

Figure 3.8 Microarraying techniques used in iSPR-biosensing. **(a)** Stealth pin contact printing technology in combination with Microgrid II spotter. The spotting is performed by dipping a pin in the ligand solution and delivering a small drop of it to the sensor surface. The pins have flat tips and defined uptake channels, which allow a thin (25 µm) layer of sample to form at the end of the pin, and printing to proceed by gentle surface contact. Printing occurs as a simple 3-step "ink-stamping" process as follows: downstroke, contact, and upstroke. Pin tips and channels are available in a wide assortment of dimensions, allowing specified spot diameter and the number of spots per loading. In this thesis SMP3 stealth pins were used, with 0.25 µL uptake volume and 0.7 nL delivery volume, producing spots with approximately 200 µm diameter on CM-dextran (SPR image shown at the bottom). Stealth pin contact spotting technology enables spot generation under humidity controlled conditions, delivering and consuming minute ligand volumes, offering densely packed microarray. However, total ligand load per spot is minute as well. Additionally, spot formation is highly dependent on the sensor surface and the immobilization buffer. The implementation of this technique to iSPR biosensing is rather limited, due to all the reasons mentioned above. Adapted with modifications from Arrayit corporation (2010)[60]. **(b)** Continuous Flow Microfluidic spotter. The Continuous Flow Microspotter (CFM) prints ligands by cycling the samples over spot surfaces and capturing them from solution. It uses a network of microchannels to print 48 ligands at one time in a 4 x 12 block from a 96 well plate. Each spot corresponds to a well in the plate and consumes 120 µL of ligand. Ligand immobilization on spot occurs in restricted environment and under flow conditions. The dimensions of the formed spots are well defined (400 x 600 µm , SPR image shown at the bottom). In comparison to stealth contact printing technology, the CFM spotter offers significantly lower throughput and consumes large samples volumes. The advantages of the CFM spotter, from iSPR application point of view, include higher ligand immobilization yield per spot, surface pre-concentration of the ligand from a dilute solution, possibility to perform different immobilization chemistries on each spot, spot formation on any surface and elimination of spot to spot cross-contamination. Adopted with modifications from WasatchMicrofluidics (2010) [61].

Table 3.1 A rough comparison of iSPR–based analysis to ELISA and LC-MS methods.

	iSPR	**ELISA**	**LC-MS**
Application	Screening		Screening and Identification
Sensitivity	Comparable		Higher
Label	No	Yes	No
Real-time monitoring	Yes	No	No
Multiplexing	High	Low	High[b]
Sample pre-treatment	None or Minimal[a]	None or Minimal[a]	Yes
Laborious	No	Yes[c]	Yes
Time consumption	Low (minutes)	Medium (one to 3 hours)	High (several hours to days)
Versatility	High	High	Low
Costs	Medium	Medium[c]	High

a- depending on the application, b- mainly for similar compounds, c-when screening for multiple targets.

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Chapter 4

Development of a Biosensor Microarray Towards Food Screening, Using Imaging Surface Plasmon Resonance.

This chapter describes the possibilities of implementing direct and competitive immunoassay formats for small and large molecule detection on a microarray, using IBIS imaging surface plasmon resonance (iSPR) system. First, IBIS iSPR optics performance was evaluated. Using a glycerol calibration curve on underivatized surface we observed high baseline variability, but uniform and robust sensitivity between hundred regions of interest. Further on, a direct immunoassay for bovine IgG detection and a competitive immunoassay for gentamicin and neomycin were developed. The direct immunoassay for bovine IgG detection in a microarray format showed poor sensitivity in comparison to the assay performed in Biacore 3000, due to low immobilization efficiency on spots. The competitive immunoassay for parallel gentamicin and neomycin detection in a microarray format displayed sensitivity in the ngmL^{-1} range, comparable with the sensitivity achieved in Biacore 3000 and in the range of maximum residue limits in milk, established in the European Union. We expect that, utilization of the IBIS iSPR system for food analysis, by screening high and low molecular weight compounds, will allow rapid and simultaneous detection of various ingredients and contaminants, providing the end-user with a detailed food profile. However, assay transfer from conventional SPR biosensors to the imaging microarray platform also presents new challenges, such as sufficient immobilization on spots, that must be addressed in future studies.

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Introduction

SPR biosensors are applied in a wide range of disciplines and become an increasingly acceptable analytical tool in general and in food analysis specifically [1-5]. The majority of the advantages offered by the SPR biosensors can be summarized to sensitive, non-invasive and continuous real time measurements. They do not require an electrical signal or any kind of labeling and are not disturbed by electromagnetic properties of the analyzed sample. SPR biosensors can provide both qualitative (epitope mapping, binding specificity, compound screening) and quantitative (concentration analysis, kinetic and thermodynamic constants) information. Usually, SPR biosensors for high molecular weight compound detection are based on direct assays, whereas inhibition assays are particularly useful for detection of small molecules. For example, they have been successfully used for specific detection and quantification of several molecules in food, e.g. vitamins, antibiotics, toxins and plant proteins [6-11]. To meet the requirements of the current "-omic era", SPR technology developed in the direction of highthroughput systems and multi-analyte measurements. One of such systems is imaging SPR (iSPR), which measures spatial refractive index changes on the surface [12-16]. There are several commercial iSPR instruments available, e.g. Biacore Flexchip (GE-Healthcare), SPRi-Plex™ (Genoptics Bio interactions), ProteOn™ XPR36 (Bio-Rad laboratories), SPRimager®II ARRAY system (GWC Technologies) and IBIS iSPR (IBIS Technologies B.V.). The instruments differ in optics, fluidics, sample handling and surface preparation. In this study we used the IBIS iSPR system, which is based on angular modulation of monochromatic plane-polarized light. In this system a 25 mm^2 surface area is illuminated with incident light at different angles and the images of the surface are captured by a charge-coupled device (CCD) camera. Light reflectivity is determined from the gray values of the pixels and plotted as a function of the scanning angle [17, 18]. Data acquired from the camera are processed automatically by the software and the response is expressed as a shift of the SPR angle in millidegrees. SPR angles are monitored simultaneously on the entire imaged surface using pre-defined regions of interest (ROIs). Fluidics on the sensor chip surface is handled by a cuvette or a flow cell. The instrument is also capable of automatic sample handling from different containers and a microtiter plate. Spatial modification of the surface, by microarraying, in combination with iSPR allows multiplexing analyses of several different compounds in a single measurement (Figure 4.1). The goal of this study was to develop a microarray biosensor using IBIS imaging SPR system and evaluate its performance and analytical applicability.

First we considered the optical sensitivity and reproducibility of the IBIS iSPR instrument. Then, we developed on the microarray biosensor platform a direct immunoassay for detection of bovine IgG (bIgG), as a model for high molecular weight compounds in food. This is particularly useful for determining milk adulteration [19]. Further on, a competitive immunoassay for detection of neomycin and gentamicin, as models for low molecular weight compounds, was developed. We used neomycin and gentamicin as an example for broad-spectrum antibiotics, commonly used in veterinary. Due to the high risk for the consumer, their presence in food is closely monitored [20].

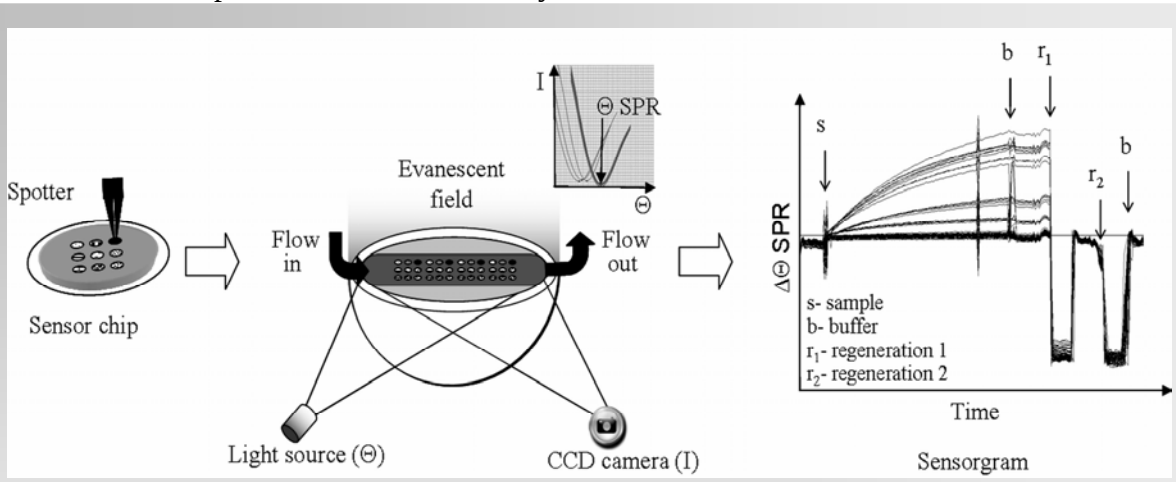

Figure 4.1 Scheme of the work flow with a microarray biosensor based on imaging SPR system. CMD sensor chip is spotted with different ligands using contact spotter. Then the sensor chip is mounted on a glass prism and assembled with the flow cell. The surface of the sensor chip is illuminated at different light angles and images of the surface are taken by a CCD camera. For each spot the SPR angle is determined from angle versus intensity plots. The change in SPR angle (sensorgram) is monitored in real time and simultaneously on all the spots during buffer, sample and regeneration solution injections.

Results and Discussion

IBIS iSPR optics performance

In order to investigate the basic optical performance of the instrument we used a template that would resemble a 100 spots microarray, by defining 100 ROIs on underivatized CMD surface. We evaluated the sensitivity and reproducibility of the IBIS iSPR instrument and compared SPR measurements obtained from individual ROIs by introducing glycerol solutions at different concentrations. First, we observed baseline variation between different ROIs, ranging from -50 to 350 millidegrees. ROIs closer to the center of the imaged surface display the highest and the lowest signals, with an average of 50 % variation between the different ROIs. Baseline variation over the surface may result from surface heterogeneity or ,alternatively, from the optical settings of the instrument. Since we used sensor chips with a gold layer coated by a CMD layer, surface heterogeneity of

both should be taken into consideration. Whereas variation in the gold layer thickness is known to influence the broadness, but not the position of the SPR dip, the surface heterogeneity must have originated from the dextran layer [2122]. However, XPS analysis of the surface showed only a small variation in the C_{1s} atomic composition (2.7 %), thus, the source of the baseline variation is probably related to the optical settings of the instrument and not to the surface heterogeneity. In spite of the observed differences in baselines, the sensitivity on each ROI was found to be uniform over the imaged surface. Glycerol calibration curves measured on each of the 100 ROIs displayed very similar slopes with high linearity to glycerol concentration ($R^2 = 0.9997$) with 1.5 % variation. Knowing that the sensitivity on each ROI is the same, the main drawback of baseline variation is that subtraction of the response on a reference spot from a response on a measuring spot does not yield reliable quantification of the immobilized amount. Descriptive figures of the results reported above are provided in the supplementary information (Figure 4.6 in supplemental information). The sensitivity of the instrument was calculated from the glycerol calibration curves and averaged over the 100 ROIs. One percent glycerol change in solution produces 140.13 millidegree shift in SPR angle. This value is close to the expected theoretical shift, 140 millidegree per Δ 1 % glycerol, which was calculated using Hansen's N-phase method adapted to four phase reflectivity calculation, see supplemented information, [23, 24]. The average sensitivity did not vary much (1 %), neither between different days, nor between different sensor chip batches (data not shown). To estimate the minimal amount of protein on the surface that is needed to produce an SPR signal in this instrument, the limit of detection (LOD) was calculated. Using 10.8 $pgmm^{-2}$ = 1 millidegree relationship, supplied by the manufacturer, the LOD was calculated to be 43 pg protein per mm^2. This conversion factor was checked by performing exactly the same glycerol experiment in Biacore 3000, using 1 RU = 1 pg protein per mm^2 relationship for comparison (data not shown) [25]. This LOD implies that there should be at least 0.96 pg of protein molecules bound to the surface in order to detect the binding on a 150 x 150 µm ROI that approximately fits a 200µm diameter spot. For IgG molecules, the distance between the binding sites at the LOD would be hundred nm within this ROI, and is eight times lower then the distance reported for the high-density immobilized CMD surface (12 nm) [26]. This indicates that the LOD of the system is sufficiently lower than the spot capacity, and thus should be suitable for bimolecular interaction measurements in a microarray format. Recently Beusink et al. reported 2.5 times higher LOD in IBIS iSPR system of 1.62 attomole (2.43 pg) IgG per 150 x 150 µm ROI [17]. The difference originates

in the procedure to determine the LOD value. Beusink et al. derived the LOD value from responses generated by binding of an anti-biotin antibody to biotinylated groups of antibodies and peptides immobilized to the surface. Although this LOD value is expressed in accumulated mass per mm^2, it is dependent on the affinity of the biomolecular interaction and immobilized ligand properties, whereas we derived the LOD value based singularly on the IBIS iSPR system properties. The LOD value presented in this paper is invariant to the ability of the surface to capture analyte molecules and thus is application independent. It should be noted that 10.8 $pgmm^{-2}$ = 1 millidegree relationship, that we used for the calculations, is true for proteins, but is expected to be different for low molecular weight compounds [27]. The summary of the IBIS iSPR optics performance is presented in Table 4.1.

Table 4.1: Summary of the IBIS optics performance.

Sensitivity	Δn 0.1429 = Δ140 mdeg
Sensitivity variation between ROIs	1.5 %
Inter experimental variability	1 %
Baseline variation between ROIs	50 %
Limit of detection (LOD)	43 pg/mm^{2} *

Δ=change; n = refractive index of solution; variation = average standard deviation/average response x100; LOD= baseline noise plus three standard deviations; *based on 1 mdeg = 10.8 pg/mm2

It is difficult to compare between currently commercially available imaging SPR sensors, due to different implementations of the technology. Nevertheless, we would like to point out two main advantages of the IBIS iSPR that in our opinion make it stand out. Firstly, the IBIS iSPR sensor may be especially useful for multi-analyte measurements in complex matrixes, such as food, because the light does not pass through the analyzed sample, as opposed to Flexchip system. Presence of the large particles in food samples is quite common and may interfere with the SPR measurements by light scattering. Secondly, IBIS iSPR system is neither restricted to a specific surface chemistry, as opposed to systems like Genoptics and Flexchip, nor to measurement at pre-defined positions on the surface, as opposed to GWC system. The flexibility of the IBIS iSPR system in terms of surface chemistry and immobilization techniques, applicable to the sensor chip, offers a broader range of applications.

bIgG detection by a microarray biosensor

We used a bIgG assay as a model system to study the performance of a direct immunoassay for detection of high molecular weight compound using a

microarray biosensor. Anti bIgG antibody was spotted on the sensor chip and responses with different concentrations of bIgG were measured. For comparison, we set up the same assay using a 4-flow channel (4FC) biosensor in Biacore 3000. In Biacore 3000, the sensor chip was first coated with anti bIgG antibody at different levels in four FCs and then allowed to react with different concentrations of bIgG. The SPR images of both sensor chips is shown in Figure 4.2.

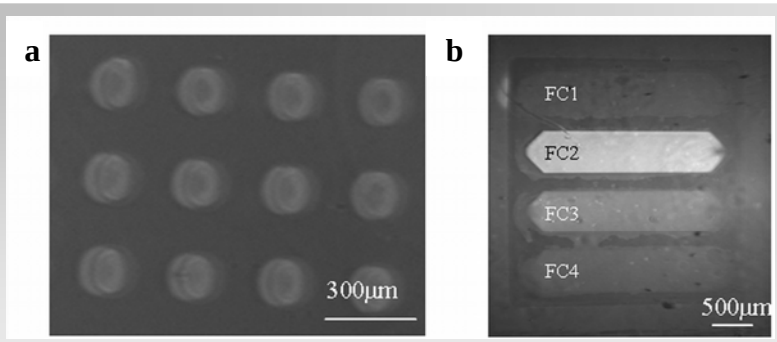

Figure 4.2 SPR images of sensor chips with immobilized anti bIgG antibody. CMD sensor chip spotted in Microgrid II with 1 mg mL^{-1} anti bIgG (**a**). CM5 sensor chip with different immobilization levels (FC1- 0 RU, FC2- 8892 RU, FC3- 2078 RU and FC4- 543 RU) of anti bIgG, coated in Biacore 3000 at 0.025 mg mL^{-1} concentration (**b**).

The responses obtained from Biacore 3000 were converted to millidegrees (10.8 RU = 1 millidegree) and plotted together with the responses obtained from the microarray in IBIS iSPR (Figure 4.3). In a microarray based assay, the responses did not increase much with increasing bIgG concentration in solution, whereas in Biacore 3000 based assay, dose response was observed in two of the flow channels. From the measurements conducted in Biacore 3000, it can be seen that the responses are decreasing with lower immobilized amounts of anti bIgG. As expected, FC2, with the highest amount of immobilized antibody, produces the most sensitive calibration curve, whereas in FC3 and FC4 the curve flattens. Additionally, from the SPR image of the sensor chips it can be seen that the intensity of the spots on the microarray is in between the intensities of FC3 and FC4, which is reflected in the responses obtained from the spots.

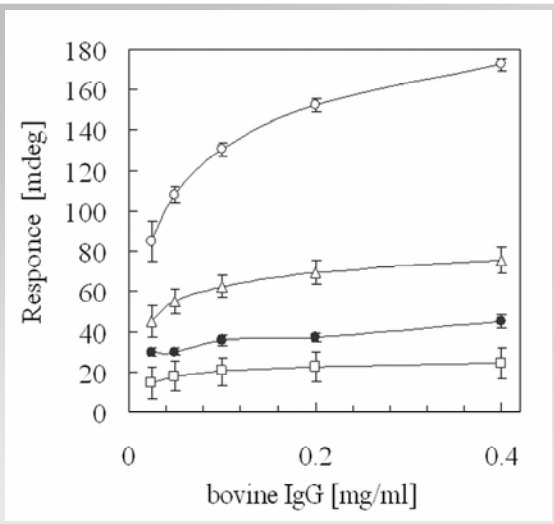

Figure 4.3 Direct immunoassay for bIgG detection performed in a microarray format using IBIS iSPR and in conventional 4FCs format using Biacore 3000. CMD sensor chip was spotted with 1 mg mL^{-1} anti bIgG and bIgG calibration curve was measured in duplicate on each spot using IBIS iSPR (black). CM5 sensor chip was coated with different levels of anti bIgG, and bIgG calibration curve was measured in duplicate using Biacore 3000 (white, FC2- round, FC3- triangle, FC4- square series).

These observations suggest that the amount of immobilized antibody on the spots is most probably the cause of the non dose dependent calibration curve. Since the concentration of anti bIgG antibody used for spotting is forty times higher than the concentration of anti bIgG antibody used for the immobilization in Biacore, it is evident that the immobilization efficiency on the microarray is significantly lower. This can be expected due to the fact that during spotting only a drop of 0.7 nL is delivered to a dry, pre-activated surface, whereas immobilization conditions in Biacore flow cell are much more favourable, including: flow over the surface, well wetted surface and higher ligand volume. Low immobilization efficiency on the spots may be overcome by increasing the molarity of the molecules in the immobilization solution; however it may be difficult with compounds of high molecular weigh, such as antibodies. These results stress the importance of sufficient immobilization of molecules per spot and its effect on the performance of microarray biosensor detection assays based on direct immunoassay format. Since many biosensor based assays for food analysis have been already developed in Biacore 3000, comparison between the assay performances in the two instruments is interesting. Although the comparison between the two systems is not straightforward and may be complicated, it can help to estimate the outcome of an assay transfer from Biacore 3000 system to IBIS iSPR. The immobilization step would probably present the biggest challenge in the assay transfer in cases similar to the described above, such as direct immunoassay.

Gentamicin and neomycin detection by a microarray biosensor

We used gentamicin and neomycin assays as a model system to study the performance of a competitive immunoassay for detection of low molecular weight compound using a microarray biosensor. A sensor chip was spotted with gentamicin and neomycin and solutions containing gentamicin and neomycin at different concentrations with the antibodies against them were injected in a serial manner. Sensorgrams measured simultaneously on gentamicin, neomycin and reference spots during injections of 1, 5 and 10 $ngmL^{-1}$ antibiotics in triplicate are shown in Figure 4.4. The sensorgrams show decreased responses on gentamicin and neomycin spots with raising antibiotics concentration in solution. The measurements performed in triplicate show good reproducibility. Responses on the gentamicin spots decrease more rapidly than on the neomycin spots, indicating higher sensitivity of the assay to gentamicin. The spikes at maximal responses, that are present in all the cycles, are caused by the double air plug, which passes through the flow cell after the sample injection has ended. Since the measurements are taken ten seconds

before the end of the injection they do not influence the results. The baseline remained stable throughout the measurements and the sensor chip was proven to be robust for 200 cycles (data not shown), measured over a period of two months. Percentage of binding was plotted against antibiotics concentration in solution to construct inhibition curves and to derive IC_{50} values (Figure 4.5). Both assays showed good sensitivity, IC_{50} of 8 ± 0.3 $ngmL^{-1}$ and 21 ± 1.4 $ngmL^{-1}$ for gentamicin and neomycin, respectively, which are five and three times more sensitive than previously described Biacore 3000 based biosensor immunoassays (40 and 70 $ngmL^{-1}$ for gentamicin and neomycin respectively) [28]. However, one measurement cycle in Biacore 3000 was much shorter: seven minutes versus 30 minutes in IBIS iSPR. Longer measurement time may be a disadvantage when a rapid screening method is required, nevertheless simultaneous detection of several compounds in one measurement may compensate for that.

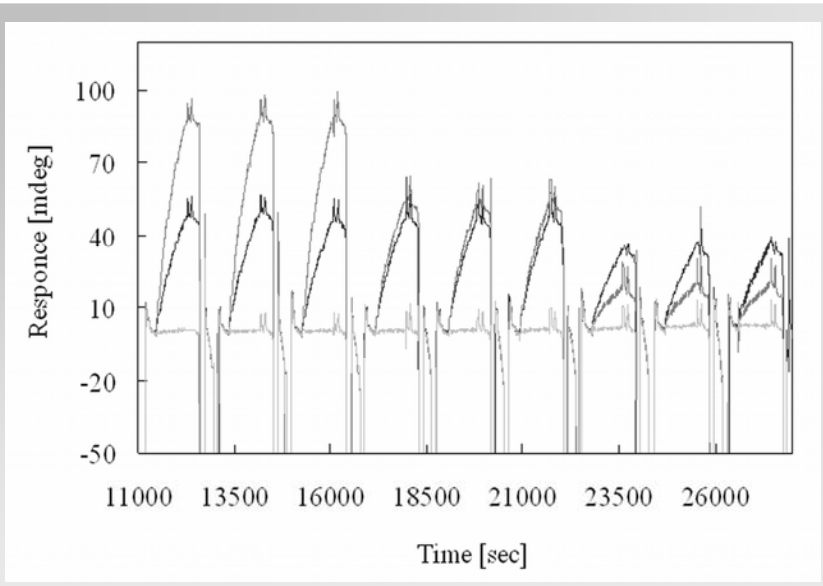

Figure 4.4 Sensorgrams recorded during competition immunoassay for gentamicin and neomycin, in a microarray format, using IBIS iSPR. CMD sensor chip was spotted with 3 mg mL^{-1} gentamicin and neomycin on different spots. Gentamicin and neomycin at several concentrations were mixed in solution with anti gentamicin and anti neomycin antibodies and introduced to the sensor chip in triplicate. Sensorgrams measured simultaneously on gentamicin (dark grey), neomycin (black) spots and a reference spot (light grey) during triplicate injections of 1, 5 and 10 ng mL^{-1} of antibiotics.

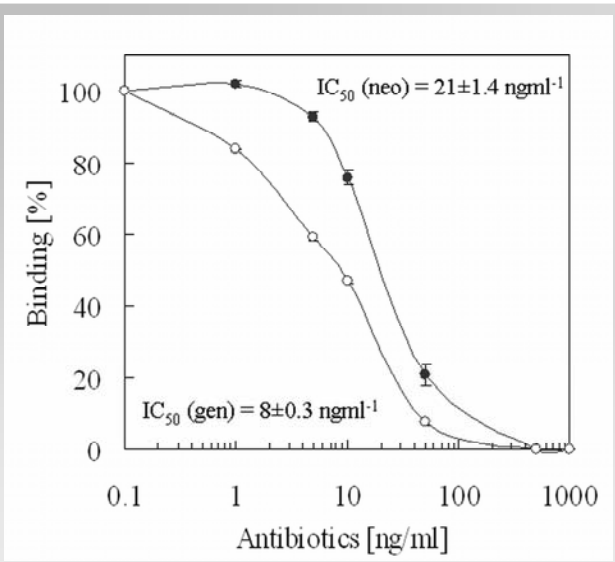

Figure 4.5 Competition immunoassay for gentamicin and neomycin detection performed in a microarray format using IBIS iSPR. Gentamicin and neomycin at several concentrations were mixed in solution with anti gentamicin and anti neomycin antibodies and introduced to the CMD sensor chip spotted with gentamicin and neomycin. Inhibition curves for gentamicin (white) and neomycin (black) were constructed from binding percentage for each antibiotics concentration and IC_{50} values were calculated.

Moreover, the maximum residue limits (MRLs) in milk, established in the European Union, are 100 $ngmL^{-1}$ for gentamicin and 500 $ngmL^{-1}$ for neomycin, requiring much lower assay sensitivity than the assay described in this paper.

Thus, in principle, the sensitivity of the assay in IBIS iSPR may be reduced, for instance, by shortening the sample injection time and by so lowering maximal response measured per concentration. Coupling of the two assays on one sensor chip did not have an effect on the assay performance; we did not observe any differences in comparison to assays conducted separately (data not shown). These results show that detection of low molecular weight compounds using competitive immunoassay work as well in microarray format with imaging SPR as in conventional SPR format. Competitive immunoassay performance is better than direct immunoassay performance in the microarray format due to the fact that it is easier to immobilize a large amount of low molecular weight ligands per spot than high molecular weight ligands.

Conclusions

Although SPR biosensors have been used in many analytical applications, commercially available imaging SPR biosensors only recently emerged on the market and, therefore, limited information about these types of biosensors exists. Prior to assay development, intrinsic sensor properties are usually studied. IBIS iSPR optics demonstrated uniform sensitivity across the microarray, suitable LOD for biomolecular interaction measurements and robustness. However, estimation of immobilized amount, under current optical settings of the instrument, was not possible due to high baseline variability. Since in concentration measurements, based on SPR biosensors, maximum load of the sensor surface is preferred, the immobilization efficiency plays a crucial role. In microarray iSPR format, immobilization of a sufficient number of molecules with high molecular weight per spot proved to be difficult, due to the spotting procedure. It was especially problematic when the detection was based on a direct immunoassay format. iSPR microarray biosensor for detection of small molecules, based on a competitive immunoassay format, was sensitive at $ngmL^{-1}$ level and robust. It displayed higher sensitivity than already established assay in Biacore 3000 and may be suitable for application in milk analysis in accordance with required MRLs. Overall, we found IBIS iSPR sensor to be a promising tool for concentration measurements. Immobilization of high molecular weight compounds in spotting format needs additional attention. Further studies will concentrate on the utilization of simultaneous multi-compound analysis in food, by combining different

immunoassays on one sensor chip. Such a device will be highly relevant for multi-analyte screening of various food contaminants and will combine the advantages of both - an SPR biosensor and a high throughput analytical system.

Experimental Section

Chemicals and Materials

Round sensor chips with a low density, 200 nm carboxymethylated dextran (CMD) layer were purchased from Xantec bioanalytics (Muenster, Germany). Biacore CM5 sensor chip, 10 mM acetate buffer pH 4, amine coupling kit containing 0.1 M N-hydroxysuccinimide (NHS), 0.4 M N-ethyl-N-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1 M ethanolamine hydrochloride pH 8.5, HBS-EP buffer containing: 10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid pH 7.4, 150 mM sodium chloride, 3 mM EDTA, 0.005 %, (v/v) surfactant polysorbate (P20) were purchased from GE Healthcare (Uppsala, Sweden). Glycerol, sodium hydroxide, hydrochloric acid, acetic acid and Tween 20 were purchased from Fluka (Zwijndrecht, The Netherlands). Affinity purified polyclonal rabbit anti bovine IgG, gamma-globulin fraction from bovine serum (bovine IgG), gentamicin sulphate powder and neomycin trisulphate powder were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Monoclonal anti neomycin and anti gentamicin antibodies were purchased from Biodesign (Huissen, The Netherlands). Round sensor chip holder, refractive index matching oil (n=1.518), hemispheric prism (BK7), cuvette and flow cell were purchased from IBIS Technologies B.V. (Hengelo, The Netherlands).

iSPR measurements

iSPR measurements were conducted using IBIS iSPR instrument. CMD sensor chip was assembled with the prism using refractive index matching oil in an appropriate holder. A flow cell (3 µL volume) or a cuvette (400 µL volume) depending on the experiment, was fixed on top of the surface. In the flow cell, the sample was delivered to a surface through a tubing and was pumped back and forth (10 $\mu Lsec^{-1}$) during the interaction. In the cuvette, the sample was delivered to the surface and mixed during the interaction by a double needle (200 $\mu Lsec^{-1}$). Prior to every measurement, the surface was equilibrated with the working buffer and ROIs in size of 150 µm x 150 µm were defined using IBIS software. The SPR angle was scanned on each pre-defined ROI in the range between -1.5 and +1.5 degrees in steps of 50 millidegrees. SPR curves were fitted automatically by IBIS software while

curve parameters were limited to 20 points before and after the dip. All the measurements were performed in the "baseline mode", recording the SPR angle as a function of time. Subsequently, SPR data were analyzed using Scrubber2 software (BioLogic Software). Final responses were calculated from the difference in SPR angle before the start and the end of sample injection and referenced to the response on a blank surface.

IBIS iSPR optics evaluation

100 ROIs were defined in the middle of the imaged surface of a CMD sensor chip representing a 10x10 microarray. The surface was wetted with RO water and baselines on each ROI were measured. To eliminate flow pattern influence on the responses, filling and emptying the cuvette was done manually and without mixing. In order to construct a calibration curve of a change in the SPR signal as a function of increasing the refractive index of the solution, glycerol solutions in the following concentrations: 0.5, 1, 2, 3, 4, 5 % (v/v) were used. Following baseline measurements, glycerol solutions were introduced to the surface and the responses on each ROI were measured. Before each new glycerol solution the fluid in the cuvette was replaced three times, to minimize the effect of the previous solution on the measurement, and the baseline with water was re-measured. All the measurements were performed in duplicate and repeated on three different days and sensor chips. The responses, measured on each ROI, were plotted as a function of glycerol percentage. From the slope of the described glycerol calibration curve we calculated the optical sensitivity of the IBIS iSPR per ROI and averaged 100 ROIs to obtain the average sensitivity over the microarray. Variation in sensitivity between ROIs was derived from the standard deviation in sensitivity and inter-experimental variation in sensitivity was derived from standard deviation in average sensitivity between experiments performed on three different days and sensor chips. Limit of detection (LOD) was calculated from the average baseline noise plus three standard deviations.

X-ray Photoelectron Spectroscopy (XPS)

The XPS analysis of surfaces was performed using a JPS-9200 Photoelectron Spectrometer (JEOL, Japan), on underivatized Xantec CMD sensor chip. The high-resolution spectra were obtained under UHV conditions using monochromatic Al Kα X-ray radiation at 12 kV and 25 mA, using an analyzer pass energy of 10 eV. $Au_{4f7/2}$ peak was used for the gold layer characterization and C_{1s} peak was used for the dextran layer characterization. The atomic percentage of C and Au was measured on twelve spots on the surface, using a 200 μm spot size, diagonally across an area of 9 mm^2 in the exact middle of the sensor disk.

Microarray manufacturing

A Xantec CMD sensor chip was activated with a freshly prepared mixture of 0.1 M NHS and 0.4 M EDC for ten minutes at room temperature. The EDC and NHS mixture was washed away with ice cold 5 mM acetic acid, the sensor chip was dried under a stream of nitrogen for four minutes and immediately spotted using Microgrid II contact arrayer (ApogenDiscoveries, UK) equipped with SMP3 Telechem stealth pins. The ligands were prepared beforehand in appropriate pH spotting buffer: bIgG in 10 mM acetate buffer pH 4.5, anti bIgG in 10 mM acetate buffer pH 4, gentamicin and neomycin in 10 mM carbonate buffer pH 8.5. The activated sensor chip was spotted with the ligands at 80 % relative humidity and was left inside the instrument for one hour after the spotting; subsequently the surface was blocked with 1 M ethanolamine pH 8.5 and rinsed with HBS-EP buffer.

bIgG detection by direct immunoassay based microarray biosensor in IBIS iSPR

Xantec CMD sensor chip was spotted with twelve spots of 1 $mgmL^{-1}$ anti bIgG as described in section 2.5. The spotted sensor chip was assembled with the cuvette in IBIS iSPR and ROIs were defined on each spot. bIgG calibration curve was constructed from responses generated by injections of different bovine IgG concentrations (0.025, 0.05, 0.1, 0.2, 0.4 $mgmL^{-1}$) in HBS-EP buffer to the sensor chip surface in duplicate. Regeneration conditions were optimized to be 100 mM HCl followed by 50 mM NaOH.

bIgG detection by direct immunoassay in Biacore 3000

Biacore CM5 sensor chip was coated with anti bIgG antibody via amine coupling procedure in Biacore 3000, as follows. Prior to immobilization, the antibody was diluted in 10 mM acetate buffer pH 4 to a final concentration of 25 μgmL^{-1} and the sensor chip was pre-conditioned by serial injections of 100 mM HCl, 50 mM NaOH and 0.5 % (v/v) Tween 20. The sensor surface was activated with a freshly prepared mixture of 0.1 M NHS and 0.4 M EDC for ten minutes, followed by injections of the antibody until the desired immobilization level was reached. Subsequently, the surface was blocked with 1 M ethanolamine pH 8.5 for ten minutes and stabilized by serial injections of 50 mM NaOH. Regeneration conditions were optimized to be 50 mM HCl injection followed by 30 mM NaOH. bIgG calibration curve was constructed from responses generated by injections of different bIgG concentrations (0.025, 0.05, 0.1, 0.2, 0.4 $mgmL^{-1}$) in HBS-EP buffer to the sensor chip surface in duplicate. The response was calculated as a difference in SPR signal (measured in RU)

before and after the sample injection. To compare between Biacore 3000 and IBIS iSPR responses, we used a conversion factor of 10.8 RU = 1 millidegree, as provided by the IBIS iSPR manufacturer.

Gentamicin and Neomycin detection by a competitive immunoassay based microarray biosensor in IBIS iSPR

A Xantec CMD sensor chip, spotted with four spots of 3 $mgmL^{-1}$ gentamicin and four spots of 3 $mgmL^{-1}$ neomycin was assembled with the flow cell in IBIS iSPR and ROI was defined on each spot. Neomycin and gentamicin calibration curves were constructed from responses generated by injections of different antibiotic concentrations, ranging from 0.02 to 2000 $ngmL^{-1}$, in HBS-EP buffer to the sensor chip surface in triplicate. To each concentration 300 fold diluted anti neomycin and anti gentamicin antibodies were added. This antibody dilution was chosen as a good compromise between maximal responses measured with each antibiotic concentration and assay sensitivity. Between each sample injection the surface was regenerated with 100 mM HCl and 50 mM NaOH. The percentage of binding for each point in the calibration curve was calculated relatively to the response measured in a sample without antibiotics. To construct a calibration curve, the binding percentage was plotted as a function of the antibiotics concentration. Half maximal inhibitory concentration (IC_{50}) was calculated from the inhibition curve using four parameter fit in BIAevaluation software (GE Healthcare).

Supplemental Information

Calculation of theoretical shift in SPR angle

Calculation of theoretical shift in SPR angle expected for Δ1% glycerol in solution, using Hansen's N-phase method adapted to four phase reflectivity calculation,

provided by Prof. Robert's M. Corn webpage (http://unicorn.ps.uci.edu/calculations/fresnel/fcform.html).

Following parameters needed for the calculation were filled in:

- Start Angle: 65 degrees
- Finish angle: 67 degrees
- Angle increment: 0.01 degrees
- Wavelength: 840nm
- Phase #1: 1.510
- Phase #2: 0.1581+5.2430i
- Thickness: 50nm

- Phase #3=Phase #4: first calculation for water -1.333 and second calculation for
  1% glycerol-1.3344.
- Thickness: 1000nm

The expected SPR angle was calculated separately for water (66.01 degrees) and 1% glycerol (66.15 degrees), and the difference was calculated to be 0.014 degrees.

IBIS iSPR optics performance

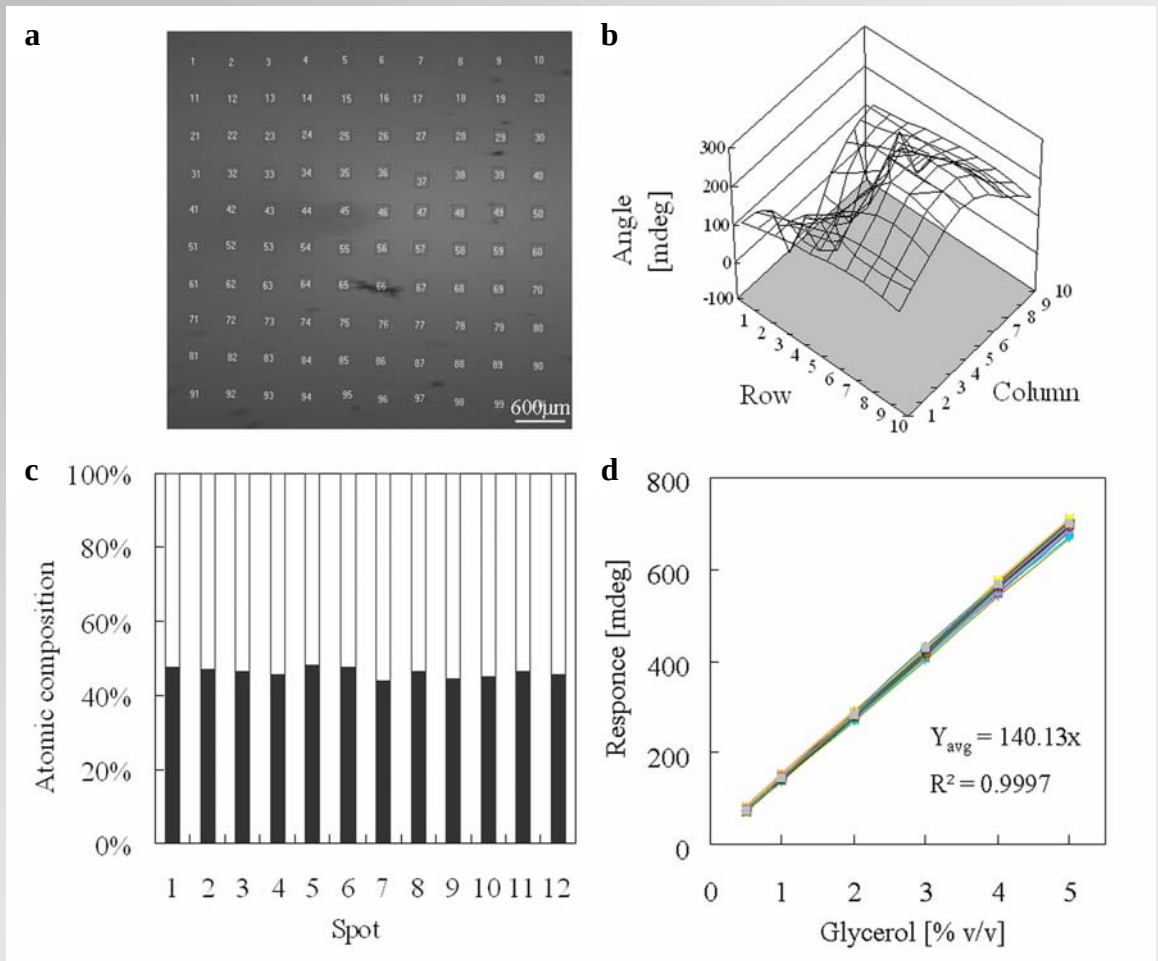

Figure 4.6 IBIS iSPR optics performance. Baseline angles with water and responses with glycerol solutions at different concentrations were measured over 100 ROIs defined on an underivatized carboxymethylated dextran sensor chip. XPS analysis of the surface was performed to establish surface composition. **a**: SPR image of the surface with 100 ROI. **b**: Baseline angles measured on each ROI. **c**: Atomic composition, C1s (white) and Au4f7/2 (black), measured from 12 different positions evenly distributed across the surface determined by XPS **d**: Glycerol calibration curves measured simultaneously on all 100 ROIs.

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Chapter 5

Label-Free and Multiplex Detection of Antibiotic Residues in Milk Using Imaging Surface Plasmon Resonance-Based Immunosensor

Monitoring of antimicrobial drug residues in food relies greatly on the availability of adequate analytical techniques. Currently, there is a need for a high-throughput screening method with a broad-spectrum detection range. This chapter describes the development of a microarray biosensor, based on an imaging surface plasmon resonance platform, for quantitative and simultaneous immunodetection of different antibiotic residues in milk. Model compounds from four major antibiotic families: aminoglycosides (neomycin, gentamicin, kanamycin and streptomycin), sulfonamides (sulfamethazine), fenicols (chloramphenicol) and fluoroquinolones (enrofloxacin) were detected using a single sensor chip. By multiplexing seven immunoassays in a competitive format, we were able to measure all the target compounds at ppb levels in buffer and in ten times diluted milk. The assays for neomycin, kanamycin, streptomycin, enrofloxacin and sulfamethazine were sensitive enough for milk control at maximum residue levels as established in the European Union. Overall performance of the biosensor was found to be comparable to that of conventional four-channel SPR-based biosensors, in terms of assay sensitivity and robustness. Combining the advantages of an SPR sensor and a microarray, utilization of the biosensor described here offers a promising alternative to the existing methods and is highly relevant for multi-analyte food profiling.

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Introduction

Antibacterial drugs are widely used in veterinary medicine to treat infectious diseases [1-3]. The fact that nearly all food-production animals receive medication for a part of their lives raises public health concerns with regards to the presence of drug residues in the food chain. Possible adverse health effects include potential allergic response (penicillins) and toxicity (chloramphenicol), but are mainly focused on a transfer of antibiotic resistance genes to human pathogens [2, 4, 5]. Even though the consumer health risks of these drug residues in foods are difficult to determine, maximal residues limits (MRLs) were established. The presence of antibiotic residues in foods is controlled by a number of national and international organizations [6]. In this study we targeted compounds, found in dairy products, belonging to four different antibiotic groups: aminoglycosides (neomycin (NEO), gentamicin (GNT), kanamycin (KAN) and streptomycin (STR)), fluoroquinolones (enrofloxacin (ENR)), fenicols (chloramphenicol (CAP)) and sulfonamides (sulfamethazine (SMZ)) (Figure 5.1).

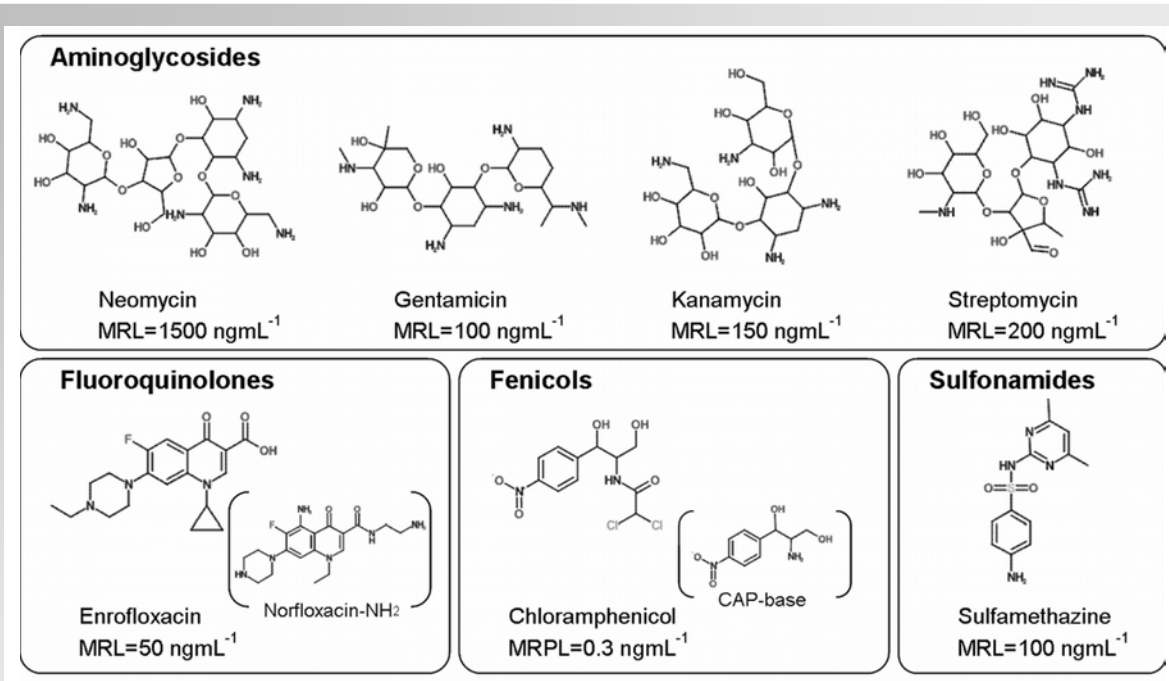

Figure 5.1 Scheme of the antibiotics measured in this study, their molecular structures and maximum residue limits (MRLs) or minimum required performance limit (MRPL) in milk. For fluoroquinolones and fenicols, molecular structures of the ligands used for immobilization on the sensor chip are showed in brackets.

In the European Union (EU), the MRLs of NEO, GNT, KAN, STR, ENR and SMZ in milk are: 1500, 100, 150, 200, 100 and 100 ng mL^{-1}, respectively [7]. For CAP, a zero tolerance policy is applied, with a minimum required performance limit (MRPL) of 0.3 ng mL^{-1} [3]. Besides consumers health concerns, the presence of antibiotics in milk is also known to have an adverse effect on the fermentation process. Therefore, dairy industry

screens incoming milk for the presence of antibiotics, to prevent contaminated milk from entering the food chain [8].

Since milk is one of the most heavily regulated products in food industry, there is a need for a single method that can simultaneously detect numerous antibiotic classes at adequate levels [8]. Traditionally monitoring methods include microbial growth inhibition assays, microbial receptor assays, enzymatic colorimetric assays, receptor binding assays, chromatographic methods and immunoassays [6, 9-12]. The milk samples are first analyzed for the presence of antibiotics using rapid and qualitative or semi-quantitive screening methods and only the suspected samples are subsequently quantified and confirmed with high pressure liquid chromatography (HPLC) and mass spectrometry (MS). In the last decade, many immunoassays have been developed (mainly in ELISA format) to provide an alternative to unspecific and time-consuming microbial growth inhibition assays. ELISAs offer quantitive detection with high specificity towards a particular antibiotic or a group of antibiotics[13]. However, when a large number of samples needs to be screened for several antibiotics, they become time-consuming and laborious as well. Thus, optical biosensors, based on biospecific interaction analysis (BIA), are of great interest, since the technology allows real time and automated analysis with relatively high capacity [14,15]. However, most of the SPR-based biosensors, developed so far, were targeting one compound or a family of structurally or functionally similar compounds, resulting in assays with a rather narrow detection spectrum [16-21]. The imaging SPR (iSPR) platform, used in this study, offers the possibility of multiplexing assays for several different compounds, making biosensor technology competitive with already existing methods [22-24]. Even though HPLC and MS are indispensable in terms of confirmation, they require expensive equipment, trained personnel and complex sample preparation steps. By using SPR biosensor-based immunoassays, one can obtain robust and quantitative results with high specificity (or broad spectrum, depending on the assay) in relatively short time. In this study we evaluated the possibility of implementing an iSPR-based biosensor for the simultaneous detection of several antibiotics in milk. The IBIS iSPR system, used in this study, was described in our previous work, where we demonstrated the possibility of high and low molecular weight compounds detection via direct and competitive immunoassay [25]. Here, seven competitive immunoassays for NEO, GNT, KAN, STR, ENR, CAP and SMZ were multiplexed on an iSPR platform and their performances were studied in buffer and in milk.

Results and Discussion

Antibiotics microarrayed sensor chip preparation

The aim of this study was to evaluate the possibility of multiplex antibiotics detection in food using an iSPR-based biosensor with milk as a model matrix. Antibiotics from four major groups were targeted: aminoglycosides (NEO, GNT, STR and KAN), fluoroquinolones (ENR), fenicols (CAP) and sulfonamides (SMZ) (Fig. 5.1).The first step in the development of an iSPR-based biosensor is a successful immobilization of target compounds on the sensor chip surface in a microarray format. When concentration assays are designed in SPR-based biosensors, maximal load of the ligand on the surface is usually preferred. Since the sensor chip should be of multiple usages in such cases, it also has to withstand multiple cycles of regeneration without major losses of immobilized ligands. We chose to covalently immobilize our compounds of interest on an amine reactive hydrogel surface using commonly applied EDC/NHS chemistry [17-19, 26, 27]. Aminoglycosides and the sulfamethazine have intrinsic primary amine groups. For the fluoroquinolones assay we used NOR-NH_2 derivative and for the chloramphenicol assay we used D-(-)-threo-2-Amino-1-(*p*-nitrophenyl)-1,3-propanediol (CAP-base) as ligands. Immobilization conditions of each compound were tested for compound solubility, spot formation on HCX hydrogel and amine chemistry compatibility (data not shown). For instance, lipophilic antibiotics requiring high percentage of DMF or dimethyl sulfoxide (DMSO) for solubility were not forming spots on the hydrogel. Furthermore, using intermediate linkers to introduce a different active group to the sensor chip surface, was not possible with the contact arraying technique, constraining us to a single immobilization step with a common chemistry of all the ligands. Thus, the initial number of antibiotics chosen for the assay development was narrowed down to the compounds described here. NEO, GNT and KAN were easily immobilized and formed well defined spots. DHS and SMZ immobilization efficiencies were lower, most likely because they have less primary amine groups and thus required higher concentrations upon immobilization. Due to the presence of DMF in NOR-NH_2 solubilization buffer, spot formation was not possible until it was sufficiently diluted (1 % (v/v) DMF final). Unlike the rest of the compounds, and in contrast to a previously published method, chloramphenicol-base was successfully immobilized in acidic buffer (pH 4.5) [19]. Antibiotics immobilization and binding of the antibodies was monitored using IBIS iSPR system, which was described in our previous work [25]. Figure

5.2A shows a representative SPR image of successfully formed spots with immobilized antibiotics and a blank spot on the sensor chip surface. Even though refractive index change caused by low molecular weight compounds is small, the spots were visible on the acquired SPR image at that stage [28].

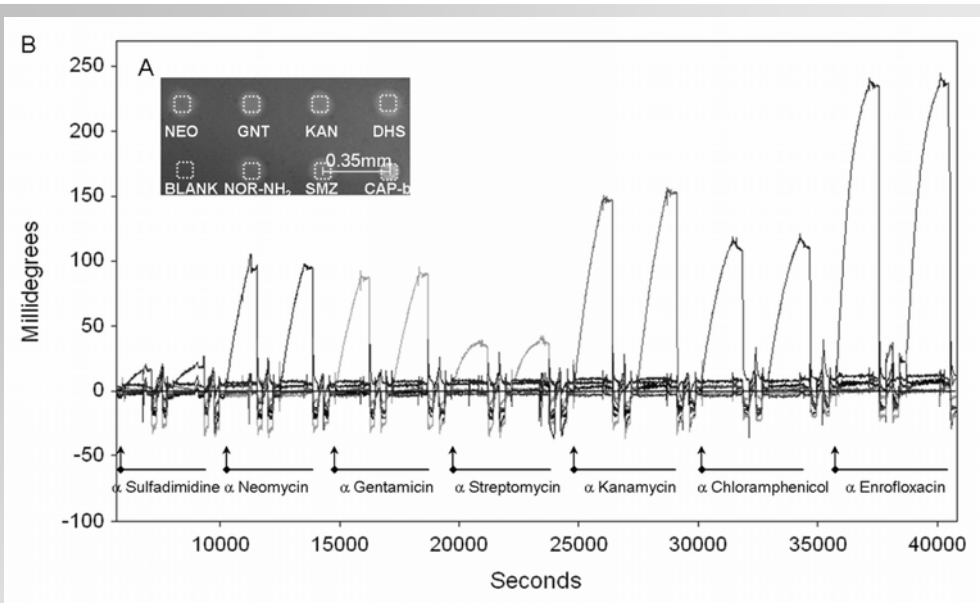

Figure 5.2 (A) - SPR image of an antibiotics microarray, acquired immediately after spotting. Neomycin (NEO), gentamicin (GNT), kanamycin (KAN), dihydrostreptomycin (DHS), norfloxacin-NH_2 derivative (NOR-NH_2), chloramphenicol base (CAP-base) and sulfamethazine (SMZ) were spotted on EDC/NHS pre-activated hydrogel sensor chip. Bar shows 0.35 mm center to center separation between spots. Dotted lines show regions of interest (ROIs), where the SPR angle was measured. **(B)** –Sensorgrams measured on seven spots containing different antibiotics during serial injections of corresponding antibodies. Injection start is marked with an arrow for each antibody duplicate. Between the antibody injections, the surface was regenerated twice with 20 % (v/v) acetonitrile in 20 mM sodium hydroxide. The sensorgrams were zeroed to the baseline before the injection and referenced to a blank spot. Alpha symbol stands for an antibody.

After several regeneration cycles, when the baseline was stabilized, the image of the spots lost its intensity, indicating removal of the compounds which were non-covalently bound to the polymer. The immobilization efficiency and spot to spot cross-reactivity were tested by serial injections of the corresponding antibodies. Figure 5.2B shows zeroed and referenced sensorgrams of these injections, measured on seven spots of different compounds. Each antibody injection caused an increase in response on the relevant spot, indicating specific antibody binding and low cross- contamination between the spots. Some cross reactivity was observed for anti-NOR and anti-CAP (less than 5 %). NOR-NH_2 and CAP-base sensorgrams also showed a small baseline build up (approximately 10 %). According to the results obtained in this experiment, final antibody dilutions were re-adjusted and an additional regeneration cycle was added in subsequent measurements. Sensor chip preparation is a crucial step in SPR-based biosensor assay development, since the quality of the modified sensor chip surface influences greatly assays sensitivity and

robustness. Even though contact printing (etc. Microgrid) is an established method for DNA, protein and low molecular weight compound microarraying, we believe that for SPR biosensor implementation in a microarray format it is rather restrictive. This technology makes it difficult, and often impossible, to combine between optimal conditions for compound solubility, spot formation on the sensor chip surface and immobilization efficiency. It also limits the high-throughput potential of the assay, due to the uniform immobilization chemistry that has to be applied for all the compounds on a single sensor chip surface. Arraying methods implementing an individual fluidic spot approach, such as Continuous Flow Microspotter (Wasatch microfluidics), might have an advantage in such cases [29].

iSPR-based immunoassays for antibiotics detection

Multiplex antibiotic detection was achieved by combining seven immunoassays on one sensor chip. The immunoassays were implemented in the competitive format, based on inhibition of antibody- binding to the antibiotic immobilized on the surface by the antibiotic in solution. The higher is the concentration of the antibiotic in the solution the lower will be the binding of the antibody to the surface, and vica-versa. Figure 5.3A shows two raw sensorgrams measured during injections of antibody and regeneration solution on a spot containing an antibiotic and on a blank spot, representing a single measurement cycle. Each measurement cycle lasted 40 minutes including the regeneration steps) and provided 24 data points (3 for each antibiotics plus 3 blanks). Final response after the antibody injection was calculated by first subtracting the baseline (B) from the response (R) and then subtracting the response on the blank spot from the response on the antibiotic containing spot. Eight zeroed and referenced sensorgrams, measured on eight spots containing different antibiotics, during single injection of the antibodies cocktail (B_0) are shown in Figure 5.3B. Due to individual assay optimization in terms of sensitivity, regeneration and antibody consumption, maximum responses varied between 10 and 100 millidegrees. The necessity of three regeneration injections, due to difficulties in regeneration of NOR-NH_2 and CAP-base spots, prolonged the assay time by 50 %. To shorten the assay time, alternative regeneration solutions were tested, including hydrochloric acid, acetic acid, formic acid, sodium chloride, DMF, DMSO, guanidine hydrochloride and ethylene glycol at different concentrations. However, none was found to be as efficient as the 20 % (v/v) acetonitrile in 10 mM sodium hydroxide (data not shown). To study the assay's performance, multi-analyte standard solutions containing NEO, GNT, STR, KAN, ENR, CAP and SMZ were

prepared both in HBS-EP buffer and in 10 times diluted milk. Standard solutions with known antibiotics concentrations (0 - 500 ng mL^{-1}), mixed with the antibodies cocktail were serially injected in duplicate over the microarrayed chip with antibiotics. Figure 5.3C shows raw sensorgrams simultaneously measured on eight spots (NEO, GNT, DHS, KAN, NOR-NH_2, CAP-base and SMZ) during serial injections of three standard solutions at concentrations of 0.16, 0.8 and 4 ng mL^{-1} (each duplicate is marked with an arrow). The duplicates showed good repeatability and the increase in antibiotics concentrations in the solution caused decrease in the responses, as expected. The differences in baselines between the spots are attributed to intrinsic optical settings of the iSPR instrument and were found not to affect the sensitivity of each spot [25].

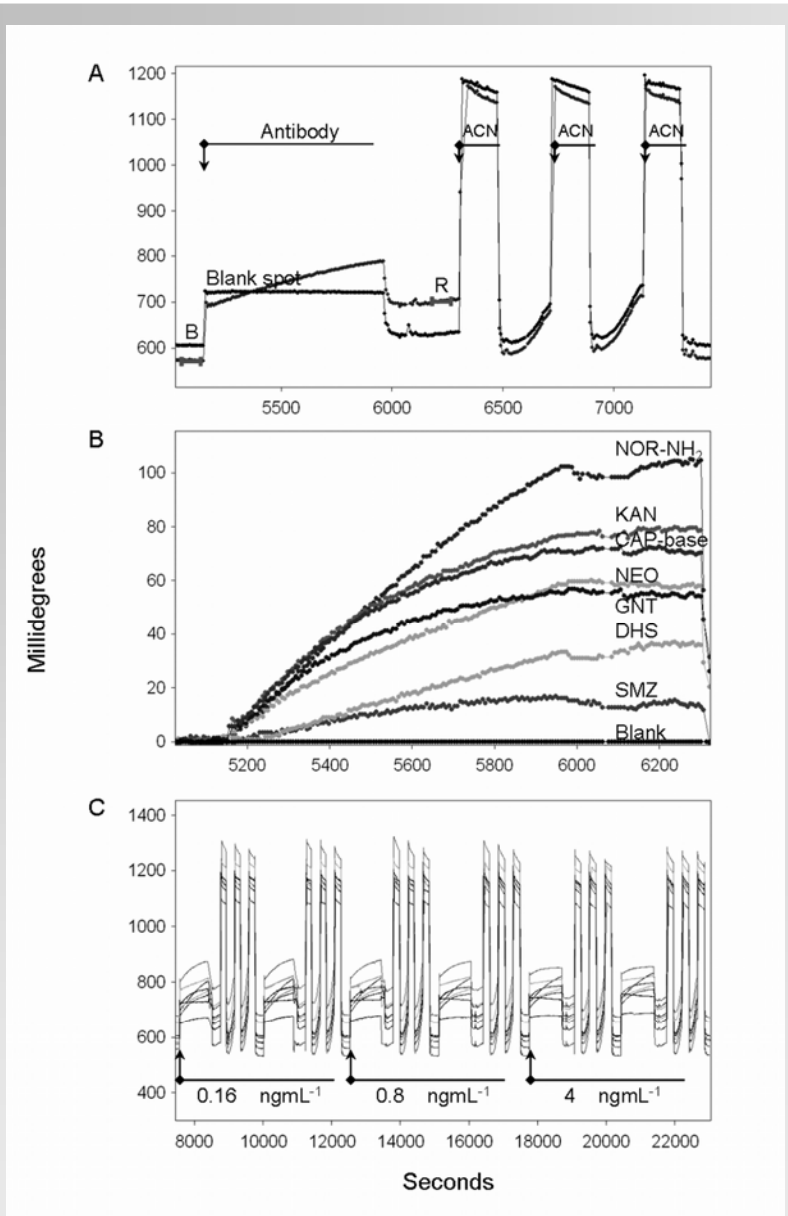

Figure 5.3 (A) – A single measurement cycle. Raw sensorgrams measured on a spot containing antibiotics and on a blank spot during antibody injection and three injections of regeneration solution (20% (v/v) acetonitrile in 20 mM sodium hydroxide). Start of each injection is marked with an arrow. Final response in each measurement cycle was calculated as follows. Baseline (B), measured before injection start, was subtracted from a maximum response (R), measured in the dissociation phase and the final response measured on the blank spot was subtracted form the final response measured on the spot containing antibiotics. **(B)** – Zeroed and referenced sensorgrams of a single injection of the antibodies cocktail, measured simultaneously on 8 spots (neomycin (NEO), gentamicin (GNT), kanamycin (KAN), dihidrostreptomycin (DHS), norfloxacin-NH_2 derivative (NOR-NH_2), chloramphenicol base (CAP-base), sulfamethazine (SMZ) and blank spots) without the presence of antibiotics (B_0). **(C)** – Raw sensorgrams measured simultaneously on NEO, GNT, KAN, DHS, NOR-NH_2, CAP-base, SMZ and blank spots during duplicate injections of multi-analyte standard solutions (0.16, 0.8 and 4 ng mL^{-1}) mixed with corresponding antibodies. Injection start of each concentration duplicate is marked with an arrow.

For each compound, calibration curves in buffer and in 10 times diluted milk were plotted using relative binding values (B/B_0) as a function of antibiotic concentration (ng mL^{-1}) (Fig. 5.4). The curves were fitted with the 4P model, and IC_{50} values were interpolated. Goodness of fit (R^2) and steepness of the curves are shown in Table 5.1. For most of the assays, the 4P model fitted the data well, both in buffer and in milk. Curves steepness of NEO, GNT and STR assays did not change much in milk, but decreased in CAP and SMZ assays and increased in KAN and ENR assays. It can be also seen, that some of the curves shifted right to the higher concentrations range and some remained unchanged (Figure 5.4).

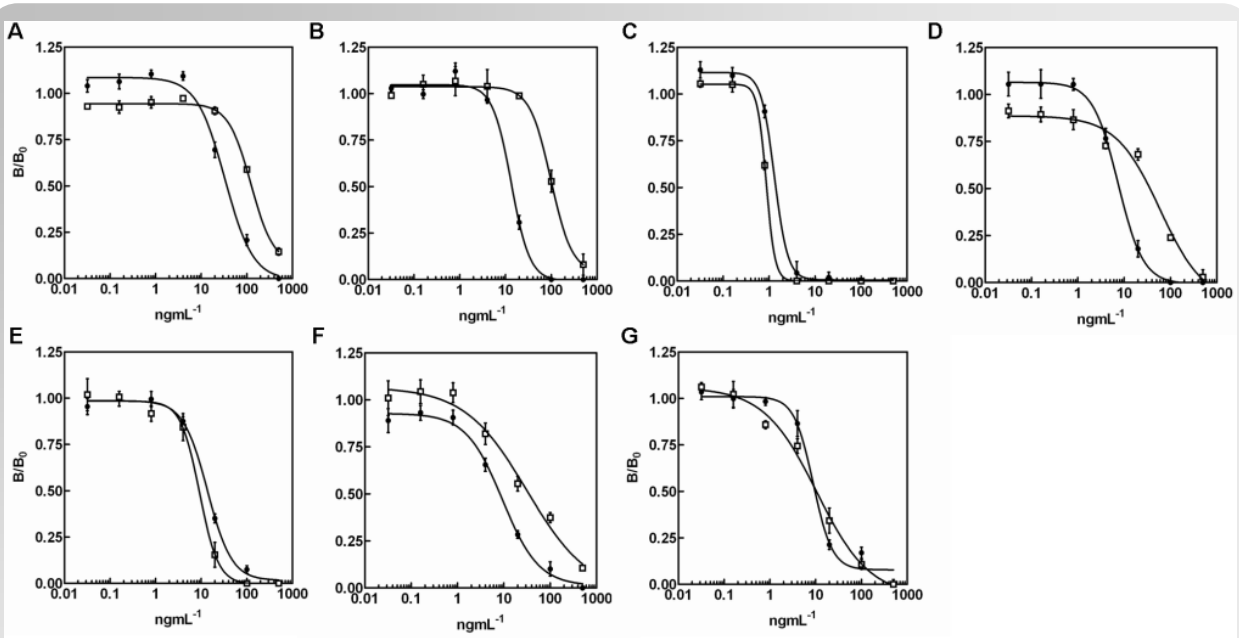

Figure 5.4 Multi-analyte calibration curves in buffer (black circle) and in 10 times diluted milk (white square) of seven antibiotics: **(A)** - Neomycin, **(B)** - Gentamicin, **(C)** – Kanamycin, **(D)** – Streptomycin, **(E)** – Enrofloxacin, **(F)** – Chloramphenicol, **(G)** – Sulfamethazine. Multi-analyte standard solutions, containing antibiotics at concentrations ranging from zero to 500 ng mL^{-1}, were mixed with a cocktail of corresponding antibodies and injected in duplicate over the sensor chip, microarrayed with antibiotics. Relative binding (B/B_0) was calculated by dividing a response (B) of each concentration by a response obtained in a solution without antibiotics (B_0). Solid lines show curves fitted with 4-parameters model. Error bars represent standard deviations between three different sensor chips.

Figure 5.5 shows a summary of the multiplexed assay's characteristics (IC_{50}, LOD and dynamic range values) in buffer and in milk. We did not observe an uniform influence of the milk on all the immunoassays, indicating that matrix effects, in this case, are most likely due to interaction of milk components with the antibodies or with antibiotics on spots. The effects of different food matrixes on the biosensor performance, as well as its applicability to raw milk samples, will be in focus of further research.

The immunosensor showed ppb-level sensitivity for the target compounds, both in buffer and in ten times diluted milk. NEO, KAN, STR, ENR and SMZ assays were

sensitive enough for antibiotic residues detection in milk at MRL levels. However, CAP and GNT assays required more than 20 MRPLs and 4 MRLs, respectively, of antibiotics to be present in milk, to produce 10 % signal inhibition. To improve CAP assays sensitivity, we tested a different antibody (rabbit Pab 426 raised against CAP-HS BSA), which was shown to be sensitive in the 0.03 to 2.2 ng mL^{-1} range in ELISA [30]. However, despite the big improvement in sensitivity ($IC_{50\ buffer}$ = 0.37 ng mL^{-1}), this antibody could not be utilized in the multiplex format due to cross-reactivity with the rest of the compounds (data not shown). Overall performance of the immunosensor, as described above, was found to be comparable to previously described Biacore 3000-based immunosensors (Table 5.1) [16-18, 31].

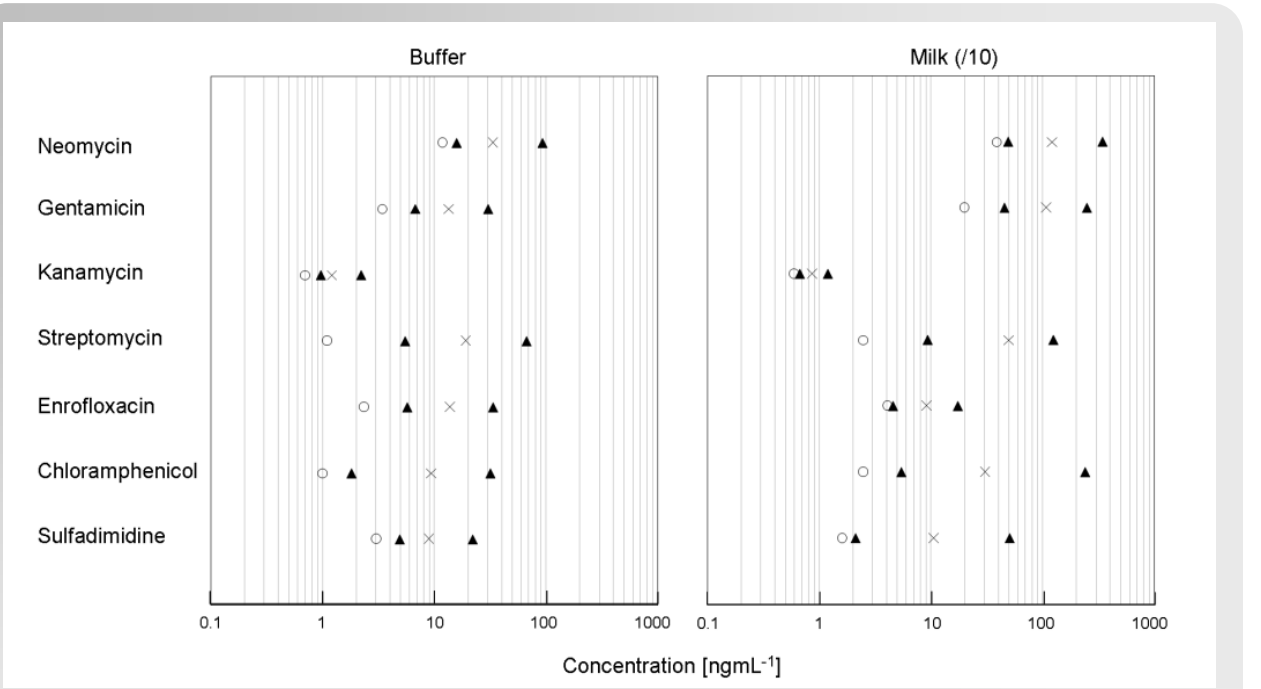

Figure 5.5 Scheme showing limit of detection (white circle), dynamic range (black triangle) and IC_{50} (x) of neomycin, gentamicin, kanamycin, streptomycin, enrofloxacin, chloramphenicol and sulfamethazine multi-analyte iSPR based biosensor assay in buffer and in ten times diluted milk. Limit of detection was calculated by subtraction of three standard deviations from the responses obtained in blank solutions without antibiotics. Assay's dynamic ranges were set between 20 and 80 % inhibition. IC_{50} values were interpolated from 4-parameters fitted curves.

Table 5.1 iSPR-based multiplex immunoassay characteristics and comparison to previously reported SPR-based immunoassays and ELISAs for antibiotics detection in milk.

		iSPR-based multiplex immunoassay				SPR – based immuno-assay	ELISA
Target compound		**Goodness of 4P-fit(R^2)[a]**	**Curve Steepness (mL ng^{-1})**	**Signal inhibition of 1 MR(P)L in milk[b]**	**IC50 (ng mL-1)[c]**	**IC50 (ng mL^{-1})**	**IC50 (ng mL^{-1})**
Neomycin	Buffer	0.9909	-1.4	56 %	33± 0.5	70 [17]	1 [33], 0.098 [34], 70 [g,35]
	Milk[d]	0.9945	-1.7		123.2 ± 0.7	150 [17]	
Gentamicin	Buffer	0.9916	-2.1	0 %	13.4 ± 1.2	40 [17]	0.92 [36], 2 [g]
	Milk[d]	0.9895	-1.8	(>3.7 MRLs)[e]	105.4 ± 15	70 [17]	
Kanamycin	Buffer	0.9971	-2.9	100 %	1.2 ± 0.2	20 [17]	2, 5 [37]
	Milk[d]	0.9992	-4.5		0.9 ± 0.07	40 [17]	
Streptomycin	Buffer	0.9892	-1.1	32 %	18.9 ± 1.2	60 [f, 17]	2 [g,35]
	Milk[d]	0.9944	-1.2		50.1 ± 1.1	140 [f, 17]	
Enrofloxacin	Buffer	0.9953	-1.6	23 %	13.7 ± 2.1	3.4 [f, 18]	2 [g,35]
	Milk[d]	0.9895	-2.1		9.2 ± 1.1		
Chloramphenicol	Buffer	0.9905	-1.1	0 %	9.3 ± 1	5.7, 2.3 [19]	0.2 [g,35]
	Milk[d]	0.9742	-0.7	(>22 MRPLs)[e]	30.3 ± 1.7	9, 4.2 [19]	
Sulfamethazine	Buffer	0.9880	-2.1	49 %	8.9 ± 1.2	8 [f, 16]	0.1 [g,35]
	Milk[d]	0.9882	-0.8		10.1 ± 1.3		

a- Goodness of the 4-parameter model fit to the calibration curve. **b**- Calculated from the 4-parameter fitted calibration curve. **c**- Half inhibitory value interpolated from the 4-parameter fitted calibration curve, averaged between three different sensor chips. **d**- In iSPR based immunoassay ten times diluted milk was used; **e**- Antibiotic concentration that should be present in milk, to produce 10 % signal inhibition. **f**- Immunoassay is based on the same antibody; **g**- Approximated value from the calibration curve presented in the product information.

In comparison to ELISA, iSPR-based immunoassays showed approximately ten fold lower sensitivity (Table 5.1) [32-34]. However, the high sensitivities of ELISA method are unnecessary for detection of antibiotics in milk at MRLs levels.

In principal, the iSPR platform used in this study can be extended to maximum of 56 multiplexed assays (using a 56-spot microarray). Use of antibodies with broad cross-reactivity range towards a family of compounds can extend the screening range even further. In our case, cross-reactivity of anti-KAN with kanamycin B and tobramycin, anti-SMZ with another 17 sulfonamides and cross-reactivity of anti-ENR with another four fluoroquinolones broadens the potential screening range of the assay to 30 antibiotic residues [18, 31].

Conclusions

In this study we showed an implementation of an imaging SPR-based biosensor to quantitative measurements of antibiotic residues in milk. Seven model compounds (neomycin, gentamicin, kanamycin, streptomycin, enrofloxacin, chloramphenicol and sulfamethazine) were simultaneously detected via multiplexed competitive immunoassay, in buffer and in milk, using one sensor chip. Neomycin, kanamycin, streptomycin, enrofloxacin and sulfamethazine assays were sensitive enough for milk control at MRL levels. The overall performance of the microarray biosensor based on iSPR was comparable to that reported for conventional Biacore-based biosensors with four flow channels. Efficient immobilization of ligands in a microarray format on the sensor chip, and sensitivity and specificity of the antibodies played a crucial role in the utilization of a multiplex immunoassay on the iSPR platform. The method described here enabled rapid, simultaneous and label free detection of model compounds from four different kinds of antibiotics: aminoglycosides, fluoroquinolones, fenicols and sulfonamides, without sample preparation, opening a door to automated and high-throughput food analysis.

Experimental Section

Chemicals and Materials

Round sensor chips, coated with pre-activated hydrogel for amine coupling (HCX) were purchased from Xantec bioanalytics GmbH (Duesseldorf, Germany). Biacore amine coupling kit (containing 0.1 M N-hydroxysuccinimide (NHS), 0.4 M N-ethyl-N-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1 M ethanolamine hydrochloride pH 8.5) and HBS-EP buffer (containing 10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid pH 7.4, 150 mM sodium chloride, 3 mM EDTA, 0.005% and (v/v) surfactant polysorbate (P20)) were purchased from GE Healthcare (Uppsala, Sweden). Twice concentrated protein printing buffer (PPBx2) was purchased from Arrayit Corporation (Sunnyvale, USA). Norfloxacin-NH_2 derivative (NOR-NH_2) was kindly supplied by dr. Sheryl Tittlemier (Health Canada, Ottawa). Monoclonal anti-neomycin and anti-gentamicin antibodies were purchased from Biodesign (Huissen, The Netherlands). Monoclonal anti-chloramphenicol and polyclonal anti-kanamycin antibodies were purchased from Abcam (Cambridge, UK). The monoclonal antibody (Mab) raised against sulfamethazine (Mab 21C7) was kindly provided by the Department of Biological Regulation of the Weizmann Institute of Science (Rehovot, Israel) [35]. The polyclonal

antiserum (Pab CA65) raised against a norfloxacin-COOH derivative was kindly supplied by Laboratoire d'Hormonologie Animale (Marloie, Belgium). Anti-DHS Mab was kindly provided by dr. Aart van Amerongen of the Agrotechnology & Food Sciences Group (AFSG) of Wageningen UR (Wageningen, The Netherlands). The full-fat goats' milk powder (Mekkermelk from Henri Willig, Katwoude, The Netherlands) was purchased locally. The iSPR instrument, round sensor chip holder, refractive index matching oil (n=1.518), hemispheric prism (BK7), cuvette and a flow cell were purchased from IBIS Technologies B.V. (Hengelo, The Netherlands). The rest of the chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Sensor chip preparation

A Xantec HCX sensor chip was spotted with ligands using the Microgrid II contact arrayer (Apogent Discoveries, Wilmslow, UK) equipped with SMP3 stealth pins (TeleChem International, Inc., Sunnyvale, USA). The ligands were prepared beforehand as follows. NEO, GNT and KAN were dissolved in water at 20 mM and immobilized in PPBx1 pH 8.5 at 10 mM final concentration. NOR was dissolved in 20 mM carbonate buffer pH 8.5 with 30 % (v/v) dimethylformamide (DMF) and immobilized in 10 mM carbonate buffer pH 9.6 at a final concentration of 0.3 mM. CAP-base was dissolved in 100 % acetic acid at 60 mg mL^{-1}, diluted to 30 mM in 10 mM acetate buffer pH 4.5 and immobilized in PPB pH 4.5 at final concentration of 15 mM. Dihydrostreptomycin (DHS) was solubilized and immobilized in 10 mM carbonate buffer pH 9.6 at 20 mM final concentration. Sulfadimidine was dissolved in 25 mM sodium hydroxide at 42 mM final concentration and immobilized directly. Each antibiotic was spotted in triplicate in randomized manner over the sensor chip surface. During the spotting, 80 % relative humidity was maintained and the sensor chip was left inside the instrument, to incubate for one hour. Unreacted groups in the hydrogel were blocked with 0.5 M ethanolamine pH 8.5, for 10 minutes at RT. If not used immediately, the sensor chip was washed with RO water, dried under nitrogen stream and stored at 4 ^{o}C.

iSPR measurements

iSPR measurements were conducted using the IBIS iSPR instrument. The sensor chip was assembled with the prism using refractive index matching oil in a round chip holder. A flow cell (3 µl volume) was fixed on top of the sensor chip surface. The sample was delivered to the sensor chip surface through a tubing and was pumped back and forth at 10 µl sec^{-1} during the interaction (10 min). The surface was equilibrated with the HBS-EP buffer (5 min) and regions of interest (ROIs) in size of 150 µm x 150 µm were defined on the spots. The SPR

angle was scanned on each pre-defined ROI in the range between - 1.5 and + 1.5 degrees in steps of 50 millidegrees (one data point every 5.5 seconds). SPR curves were fitted automatically by IBIS software while curve parameters were limited to 20 points before and after the dip. All the measurements were performed in the "baseline mode", recording SPR angle as a function of time. Subsequently, SPR data were analyzed using Scrubber2 software (BioLogic Software, Campbell, Australia). Raw sensorgrams were first zeroed to the baseline of the buffer before the injection and then referenced to a response of the blank spot (without antibiotic). The maximum responses were calculated for each injection from the data points collected during the dissociation phase (four minutes after sample injection stopped).

iSPR-based multiplexed competitive immunoassay

Seven competitive immunoassays for NEO, GNT, KAN, STR, ENR, CAP and SMZ were multiplexed as follows. A freshly prepared sensor chip, microarrayed with antibiotics as described in the sensor chip preparation section, was conditioned with at least three serial injections (two minutes contact time each) of regeneration solution containing 20 % (v/v) acetonitrile in 10 mM sodium hydroxide until the baselines of all the spots were stable in HBS-EP buffer. To test spot to spot cross-contamination and antibodies cross-reactivity, each antibody was injected separately (10 minutes contact time) in duplicate over the surface of the pre-conditioned sensor chip. From this step, a final antibody dilution was selected according to the obtained responses, considering sufficiently high response without baseline build up. Next, multi-standard solutions containing all the antibiotics mentioned above were prepared in HBS-EP buffer at concentrations ranging from 0.032 to 500 ng mL^{-1} and mixed with a cocktail of antibodies containing anti-NEO (1:500), anti-GNT (1:500), anti-KAN (1:5000), anti-STR (1:100), anti-NOR (1:500), anti-CAP (1:7000) and anti-SMZ (1:2000). These mixtures were injected over the sensor chip arrayed with antibiotics in duplicate, starting with blank solution containing only the antibody cocktail. Each cycle included sample injection (10 minutes contact time) and three injections of regeneration solution (two minutes contact time each). For measurements in milk, 1 g of milk powder was dissolved in 9 mL of HBS-EP buffer, stirred for 0.5 hr at RT, diluted ten times in HBS-EP buffer and filtered through 0.45µm HT Tuffryn acrodisc syringe filter (Pall Life Sciences, UK). Multi-standard solutions in diluted milk were prepared and measured in the same way as in buffer on the same sensor chip. Relative binding (B/B_0) was calculated by dividing the response of the antibiotics containing solution (B) by the the response of

the blank solution (B_0). To generate calibration curves, B/B_0 values were plotted against antibiotic concentrations. The calibration curves were fitted with a non-linear 4-parameters (4P) model using GraphPad Prism software (GraphPad Software, Inc) and half maximal inhibitory concentration (IC_{50}) was interpolated. Additionally, immunoassays were characterized by the limits of detection (LODs) which were calculated by subtracting three standard deviations from the average maximum response of the blank solution and by the dynamic measurement ranges, which were set between 0.2 and 0.8 B/B_0.

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Chapter 6

Food Allergens Profiling with Imaging Surface Plasmon Resonance-based Biosensor

Food allergy is a growing health concern, which currently affects approximately 4 % of adults and 8 % of infants. For consumer protection purposes, food producers are required by law to disclose on the product label whether a major allergen is used during the production process. The commonly employed monitoring methods are highly laborious, time consuming and often expensive when screening for multiple allergens. This chapter describes utilization of imaging surface plasmon resonance (iSPR) in combination with antibody array for rapid, quantitative, and multi-analyte food allergens detection. We demonstrate how the use of this technology provides a complete allergen profile within short measurement time and with adequate sensitivity. The successful applicability of this approach was demonstrated by analyzing real food products and by comparing their hazelnut content with ELISA. Our newly developed method opens the door to automated and high-throughput allergen analysis, which will ultimately provide the consumer with safer food.

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Introduction

Millions of people experience allergic reactions to food, presenting mild to life-threatening symptoms. Food allergy is also considered to be the leading cause for outside hospital anaphylaxis. So far, there is no cure for food allergy, and the only way to manage the health risk is by strict avoidance of the offending allergen. Even so, one of every four food allergic individuals suffer from allergic reaction due to accidental exposure [1, 2]. For consumer protection, legislation requiring a mandatory declaration of allergenic foods has been put into place both in EU and in the USA [3, 4]. However, this legislation refers only to ingredients that are deliberately introduced to the product, leaving out food contamination with allergens during the production process. Since processed food contains multiple ingredients and shares storage and production facilities, an allergen–free end product is difficult to guarantee. A precautionary labeling is voluntary implemented by the manufacturers to indicate possible allergens presence at trace levels.

Food allergens are abundantly occurring proteins and many foods contain multiple allergens at variable amounts. For instance, peanut contains several allergenic proteins which belong to seed storage globulin family [5-7]. Detection methods based on polyclonal antibodies usually target the total protein extract of the offending food, whereas detection methods based on monoclonal antibodies target one specific allergen or another protein as a marker [8, 9]. traditional allergen monitoring techniques include protein-based methods in various formats (immunoblotting, enzyme or radio-allergosorbent test, rocket-immuno electrophoresis and etc.) and DNA-based methods (PCR and real-time PCR) [10, 9]. All of the methods mentioned above are too time consuming and labor intensive to enable a thorough allergenic profiling with the desired confidence level. Moreover, for some allergens the methods are not readily available and/or expensive. Currently, ELISA is the only technique routinely used for food allergen analysis. Even in its fastest format ELISA requires at least 30 minutes for 14 samples to be analyzed for a single allergen at an approximate materials cost of 15 euros per sample. The highest multiplex in ELISA achieved so far included 5 allergens [11]. Considering the required sample dilutions, replicates and manual labor, screening multiple samples for multiple allergens becomes fairly unrealistic and therefore only few selected samples are analyzed for specific allergens. To fulfill the needs in rapid and multiplex analysis, several biosensors were developed for food allergen screening. The multi-analyte systems reported so far include the highly sensitive (ppb levels) NRL array biosensor, which was applied to detect ovalbumin in pasta and Biacore Q sensor, applied for several food allergens detection [12,13].

Even though significant reduction in analysis time and good sensitivities were achieved, none of them demonstrated simultaneous analysis above four allergens. Lack of adequate analytical tools for multiplex allergen detection leads to difficulties in consumer protection driven legislation and falsely labeled products. The latter, either endangers sensitive consumers or unnecessary narrows down their nutritional choices. There is an evident need for a rapid screening device with multi-analyte diagnostic facilities, which can provide a detailed food profile, enabling improvement in food safety and quality monitoring.

Here we utilised iSPR technology for rapid and quantitative allergen detection as a novel approach to food profiling. While SPR is widely applied for kinetic studies of biomolecular interactions, its application for routine concentration analysis has been limited due to high costs of the dedicated instrumentation and low-throughput. iSPR platform, on the other hand, offers multiplex analysis in a single measurement by combining SPR-based detection with spatial modifications of a surface, such as microarrays [13-19]. In this study we constructed an antibody microarray directed against 13 major food allergens on a hydrogel–coated SPR chip and applied it to label-free and direct allergen detection in food, using an angle scanning iSPR system (Figure 6.1a).

Results and Discussion

Biosensor analytical performance evaluation

For antibody microarray fabrication, hydrogel-coated SPR chips and a continuous flow microfluidic (CFM) spotter were used. The CFM spotter applies a microfluidic interface to enable antibody immobilisation on each spot individually, offering many advantages over the conventional spotting techniques, including high-quality spot formation on hydrophilic surfaces and a substantial increase in the spot load [20]. Even if only a fraction of immobilized antibodies is active towards the analyte, the response will be sufficient. This enables direct spotting of polyclonal antibodies without prior affinity purification. In this study the panel of antibodies was chosen according to the designated food product group - cookies and chocolates, and included both monoclonal and polyclonal antibodies. Seven major allergens - peanut, milk, lupine, soy, egg, hazelnut and almond, and six additional tree nut allergens- cashew nut, brazil nut, pine nut, pecan, macadamia nut and pistachio nut were targeted (Figure 6.1b). Every spot on the chip surface essentially is a specific sensing region for a particular allergen. For each antibody spot a dose response curve with the specific allergen

was constructed (Figure 6.1c). Each measurement cycle (including chip stabilization, interaction with the sample and chip regeneration) produced quantitative data on the concentration of 12 allergens within 12 minutes (Figure 6.1c).

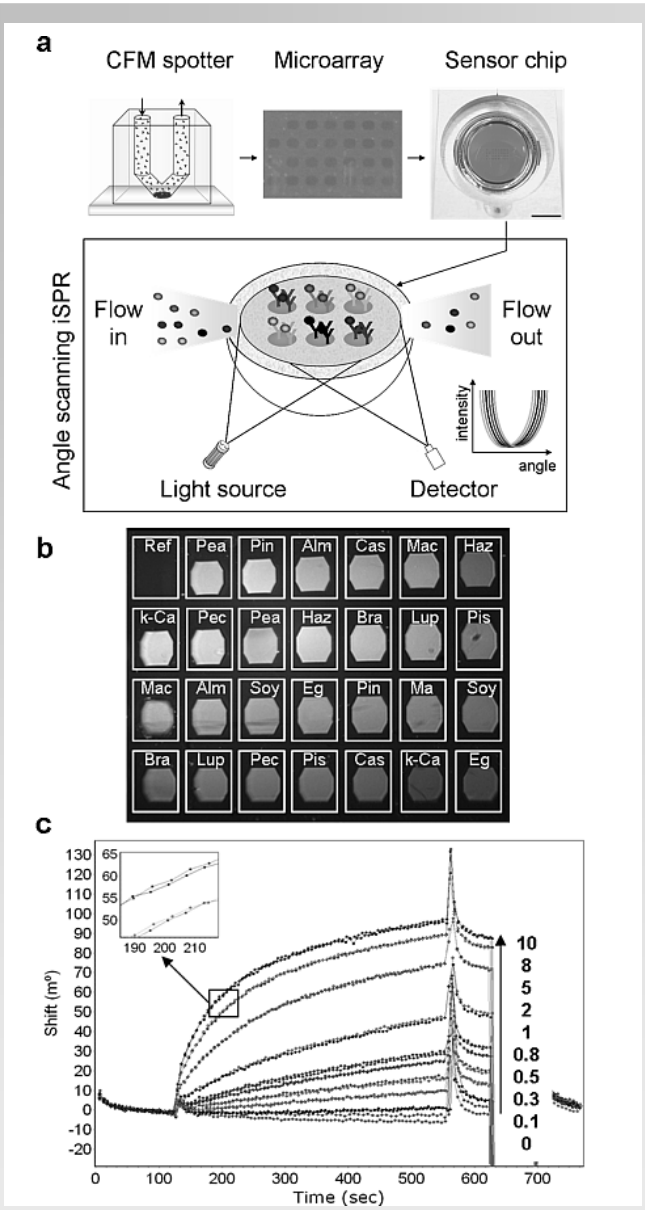


Figure 6.1 On-chip direct allergen screening using imaging Surface Plasmon Resonance (iSPR). (a) Principle of the allergen detection using iSPR system. Hydrogel coated SPR chip is spotted with antibodies against allergens using a continuous flow microfluidic (CFM) spotter. The antibody-microarrayed chip is mounted on a glass prism, assembled with the flow cell and placed in the iSPR instrument. The surface of the chip is illuminated at different light angles and images of the surface are taken by a CCD camera. For each spot the SPR angle is determined from angle versus intensity plots. The sample is delivered to the chip using a flow cell, present allergens bind to spotted antibodies and a shift in the SPR angle occurs. Scale bar, 1 cm. (b) SPR image of the microarrayed chip with anti-peanut (Pea), anti-pine nut (Pin), anti-almond (Alm), anti-κ-casein (κ -Ca), anti-macadamia (Mac), anti-hazelnut (Haz), anti-pecan (Pec), anti-brazil nut (Bra), anti-lupine (Lup), anti-pistachio nut (Pis), anti-cashew nut (Cas), anti-egg (Egg), anti-soy (Soy) and buffer (Ref). Spot dimensions are 400 x 600 µm. (c) The shift in SPR angle is monitored in real time (sensorgram) on anti- κ -casein spot during injection of: buffer (0-120 sec), sample (120-540 sec), buffer (540-660 sec) and regeneration solution (660-720 sec). Standard solutions of κ -casein at different concentrations (0-10 µg mL^{-1}) were injected in duplicate over the antibody-microarrayed chip. Zeroed sensorgrams measured on κ -casein spot are shown. The inset shows a close up on duplicate injections of 10 and 8 µg mL^{-1} κ -casein.

Total protein extracts of the selected allergenic food were used as standards to study the sensitivity and the selectivity of the chip. Multi-analyte standard solutions (containing extracts of all targeted allergens) in a concentration range from 0.1 to 10 µg mL^{-1} were injected over the antibody-microarrayed chip. For each allergen, a calibration curve was constructed by plotting the maximum binding response as a function of the concentration. Calibration curves obtained for all allergens showed dose dependency on allergen concentration, even though with different sensitivities. Most of the curves showed that the optimal working range of the chip is below 2 µg mL^{-1}, similarly to the

allergen assay described by Yman et al, suggesting a relevant analytical capability for food allergen detection (Figure 6.2a and 6.2b) [13]. Optimal curve fitting was obtained using a non-linear one-phase association model, making use of the entire concentration range (Figure 6.2a and 6.2b and Table 6.1 in supplementary information).

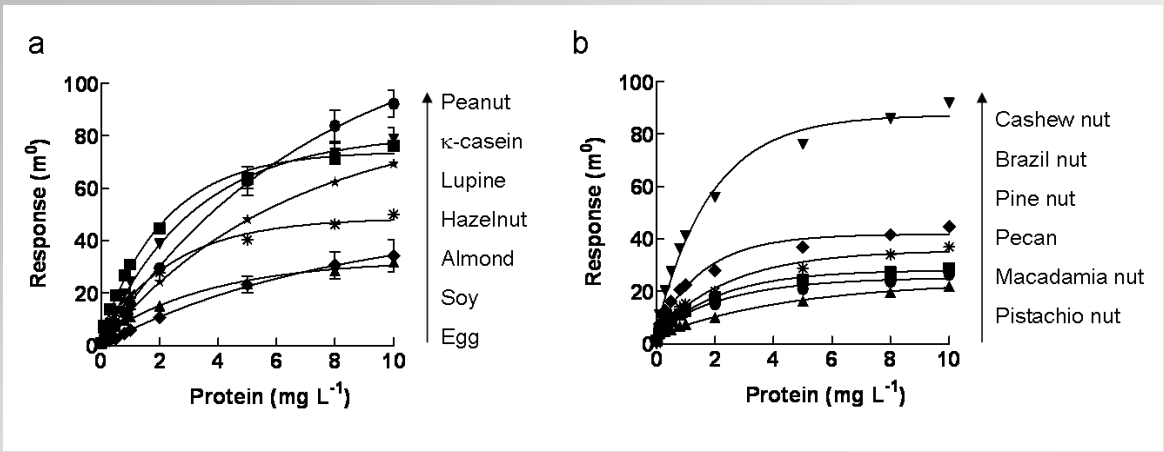


Figure 6.2 Multi-analyte calibration curves in buffer. **(a)** and **(b)** Dose response curves of 13 allergens: peanut (circle), κ-casein (reverse triangle), lupine (square), hazelnut (star), almond (asterisk), soy (diamond), egg (triangle), cashew nut(reverse triangle), brazil nut (diamond), pine nut (asterisk), pecan (square), macadamia nut (circle) and pistachio nut (triangle) measured on a single antibody-microarrayed chip using iSPR. Multi-standard solutions containing all the allergens were prepared in HBS-EP buffer at concentrations ranging from 0 to 10 µg mL^{-1}. These allergen mixtures were injected over the antibody-microarrayed chip and maximal binding responses were measured at 480 seconds after sample injection. To generate calibration curves, maximal binding responses were plotted against allergen concentration for each antibody. Solid lines show curves fitted with a non-linear one-phase model. Error bars represent standard deviations (n=4).

Next, the selectivity of the chip was assessed. Detection of a single allergenic protein in food products, which contain a large variety of other proteins at much higher amounts, requires an extreme specificity of the antibody used. However, antibodies directed against a specific protein might also bind, mostly to minor extent, to other proteins, presenting so called cross-reactivity. This cross-reactivity might cause a false positive test result in a screening assay for a single allergen. In multiplex assays however, the combination of antibodies with overlapping specificities and variable degree of selectivity is often beneficial due to the fact that it provides a greater confidence level in positive results and reduces the number of false negatives during the screening process. In this study the cross-reactivity (CR) of each antibody was assessed by injecting each allergen separately to the antibody-microarrayed chip and measuring binding responses on all the antibody-containing spots. CR was expressed as the ratio (in %) of non-specific binding (with any allergen) to specific binding (with the target allergen) at the same concentration (Figure 6.3).

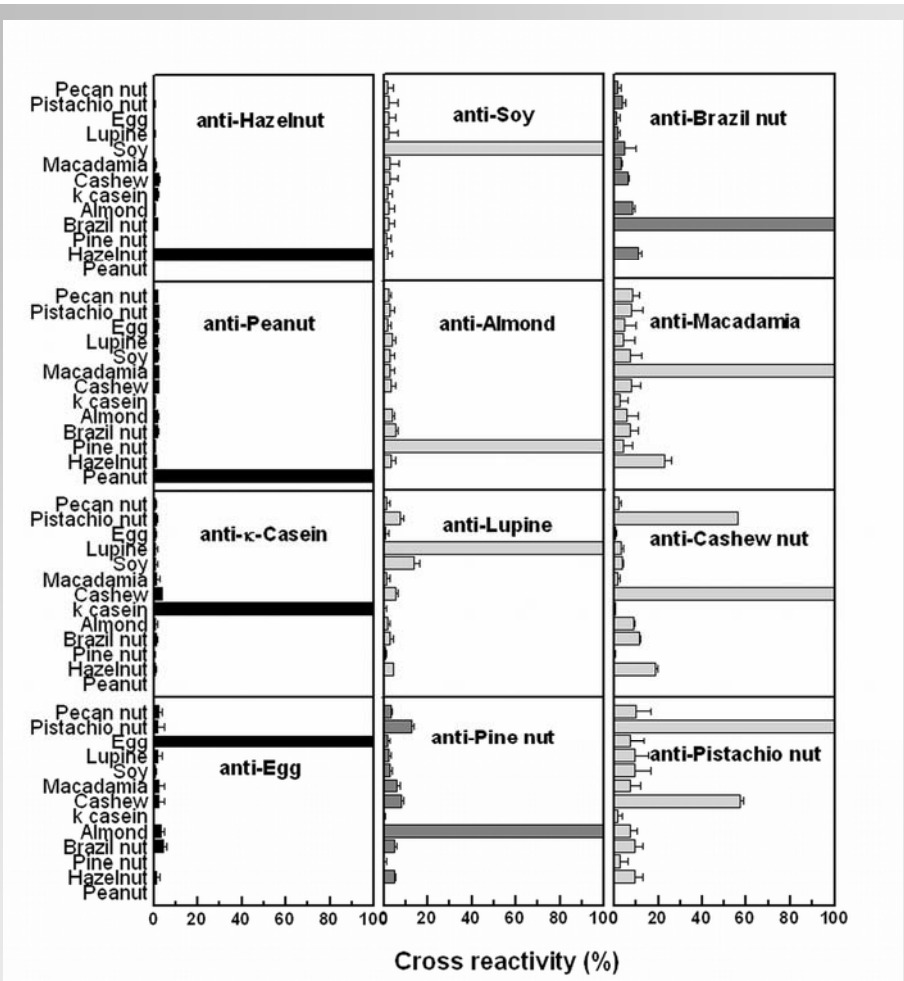


Figure 6.3 Cross-reactivity of the antibodies towards different allergens. Standard solutions containing each allergen separately were prepared in HBS-EP buffer at concentrations of 5 µg mL^{-1} and injected over the antibody-microarrayed chip. Maximal binding responses were measured at 480 seconds after sample injection and compared to the maximal binding response of the specific antigen at the same concentration (100 %). Shades of black indicate the specificity of the antibody, from most selective (black) to the least selective (light grey). Error bars represent standard deviations (n=3).

The results showed that peanut, κ-casein, egg and hazelnut were detected with highest specificity, displaying less than 1 % CR with other allergens. Lupine, soy, almond as well as pine, brazil and macadamia nuts were detected with moderate degree of CR to other allergens. The cashew nut and pistachio nut antibodies were found to be highly cross reactive with each other's allergens and the pecan antibody exhibited extensive degree of binding to all the allergens tested, except for k-casein and peanut, and thus was used for positive control as a generic binder (Figure 6.1 in supplemental information). Of course, the antibodies panel may be altered in accordance to a specific analytical need. When looking at signal stability and reproducibility, the chip was found to be highly robust. Over 200 measurements were conducted before reduction in responses occurred (Figure 6.2 in supplemental information). For example, Figure 6.4 shows the responses of lupine and hazelnut antibodies during seven calibrations curve repetitions each including 20 measurement cycles.

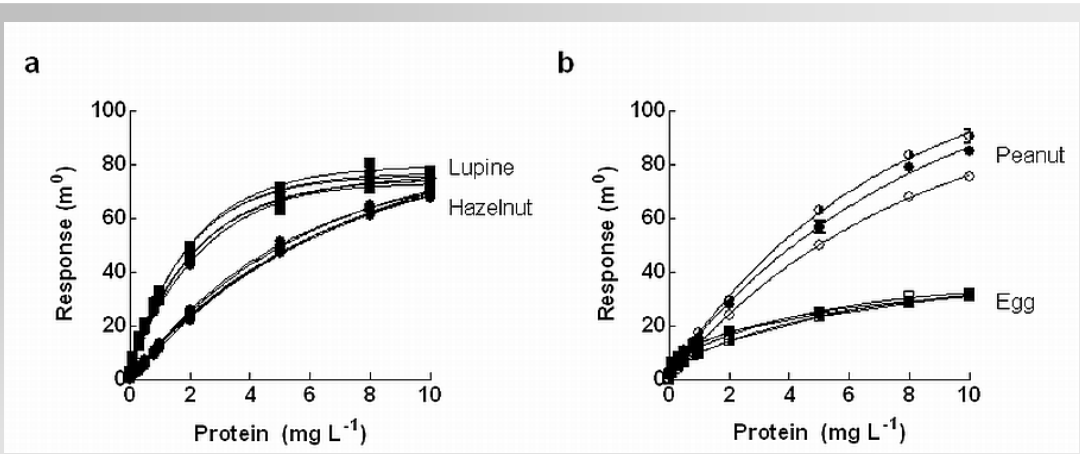


Figure 6.4 Sensor chip stability and food matrix effect **(a)** Seven calibration curves, each including 20 injections, measured on anti-lupine (square) and on anti-hazelnut (circle) spots are shown. Solid lines show curves fitted with a non-linear one-phase association model. **(b)** Peanut and egg calibration curves in buffer (black and white), in cookie extract (black) and in dark chocolate extract (white). Solid lines show curves fitted with a non-linear one-phase association model. Error bars represent standard deviations (n=4).

Food allergens profiling with iSPR biosensor

Food matrices are often complex mixtures, containing a large variety of molecules which could mask the presence of the allergen either by lowering allergen extraction efficiency or by interfering with the analytical assay. The ability to measure in food extracts is of utmost importance to the method's applicability, and was studied as follows. The allergen multi-analyte standards were spiked into cookie and dark chocolate extracts and injected over the antibody-microarrayed chip. The calibration curves were constructed in the same manner as in buffer measurements. Expectedly, the introduction of the food matrix caused reduction in binding of some antibodies, but did not significantly affect the sensitivity. For example, egg measurements remained unchanged; but the peanut antibody produced somewhat lower responses in dark chocolate in comparison to buffer (Figure 6.4). Overall, the effect of the food matrix on the analytical performance was minor, most likely due to a proper extraction method used for sample preparation (Figure 6.3 in supplemental information). This observation is especially significant considering that the measurements are based on monitoring direct binding of the allergens in the food extract to the chip without any additional steps, in contrast to ELISA which includes multiple washing, labelling and colour development steps. The sensitivity of on-chip allergen detection, expressed in limit of detection (LOD) and limit of the quantification (LOQ) of allergen protein in the food sample, was found to be in the low μg g^{-1} range both for cookies and dark chocolates, adequately compatible with food allergens analysis and comparable to most commercially available ELISAs and Biacore–based assays (Figure 6.5a) [13].

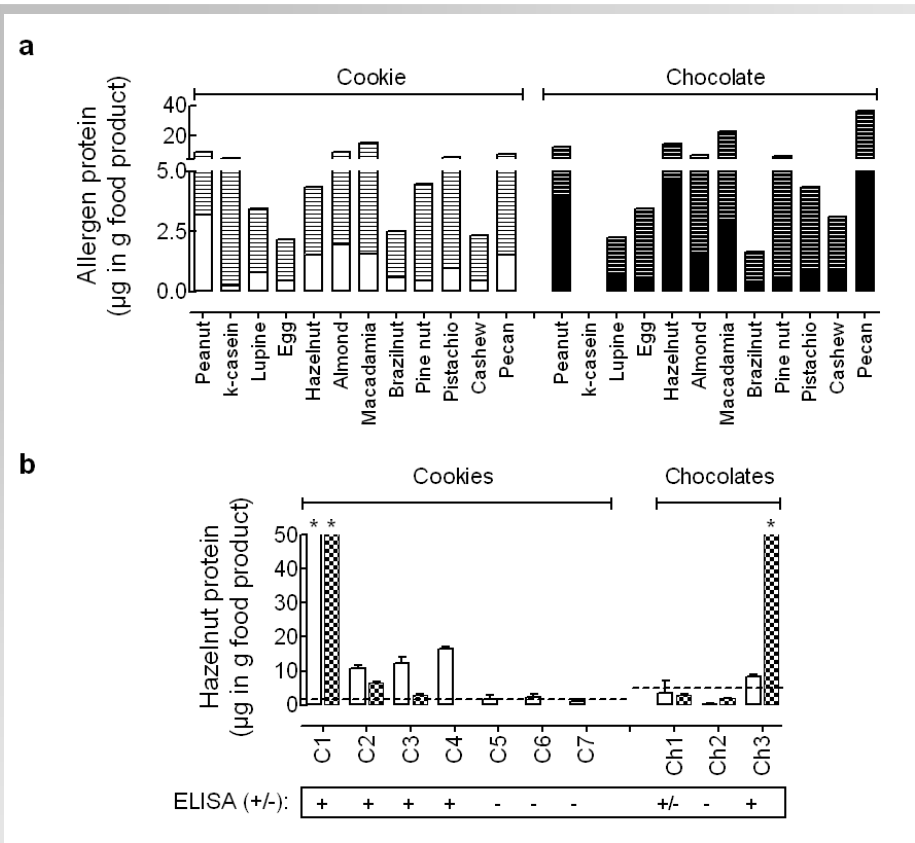


Figure 6.5 Sensitivity of the iSPR biosensor and comparison to ELISA. **(a)** Superimposed bar graph of limits of allergen detection and quantitation in cookie and in dark chocolate. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated by adding three or ten, respectively, standard deviations to the average maximum response of blank cookie samples and blank dark chocolate samples. LOD and LOQ in cookie (white and white with stripes). LOD and LOQ in dark chocolate (black and black with stripes). **(b)** Comparison to qualitative and quantitative ELISA. Seven cookies and three chocolate samples were screened for hazelnut protein content using on-chip direct measurements with iSPR (white bars) and the results were compared to results obtained previously with qualitative (+/-) and quantitative ELISA (plaid bars). Dotted lines represent LOD values for iSPR. Asterisk indicates hazelnut concentration more than 50 µg per g food product. Error bars represent standard deviations (n=4).

The applicability of the iSPR-based allergen screening was validated by analysing commercially available food samples, seven cookies and three dark chocolates, which were previously used in an EU survey for hazelnut and peanut presence assessment [21]. For comparison, all samples were also analysed for hazelnut content with an in-house qualitative ELISA and a commercially available, AOAC certified ELISA kit (Figure 6.5b). The results obtained with direct on-chip measurements correlated well with the results obtained with both ELISA assays. All samples that were positive for hazelnut in ELISA showed positive responses when analysed with our chip, and no false negatives were observed. However, hazelnut concentration in food samples determined by quantitative ELISA were somewhat lower in cookies and higher in chocolates than the values that were obtained with our chip (Figure 6.5b). The variation between the two might be attributed to the different antibodies used, as well as to the immunoassay format. In ELISA, a sandwich immunoassay format is implemented, where as in this study direct measurements were performed. Moreover, since there is neither certified reference

hazelnut material available nor a method for absolute allergen concentration determination, it is difficult to judge which value is the correct one. As long as the concentrations obtained with both methods are in the same order of magnitude the values are generally considered to be comparable.

The allergenic profile obtained with direct measurements on the chip revealed different fingerprints for each food sample (Figure 6.6a and 6.6b).

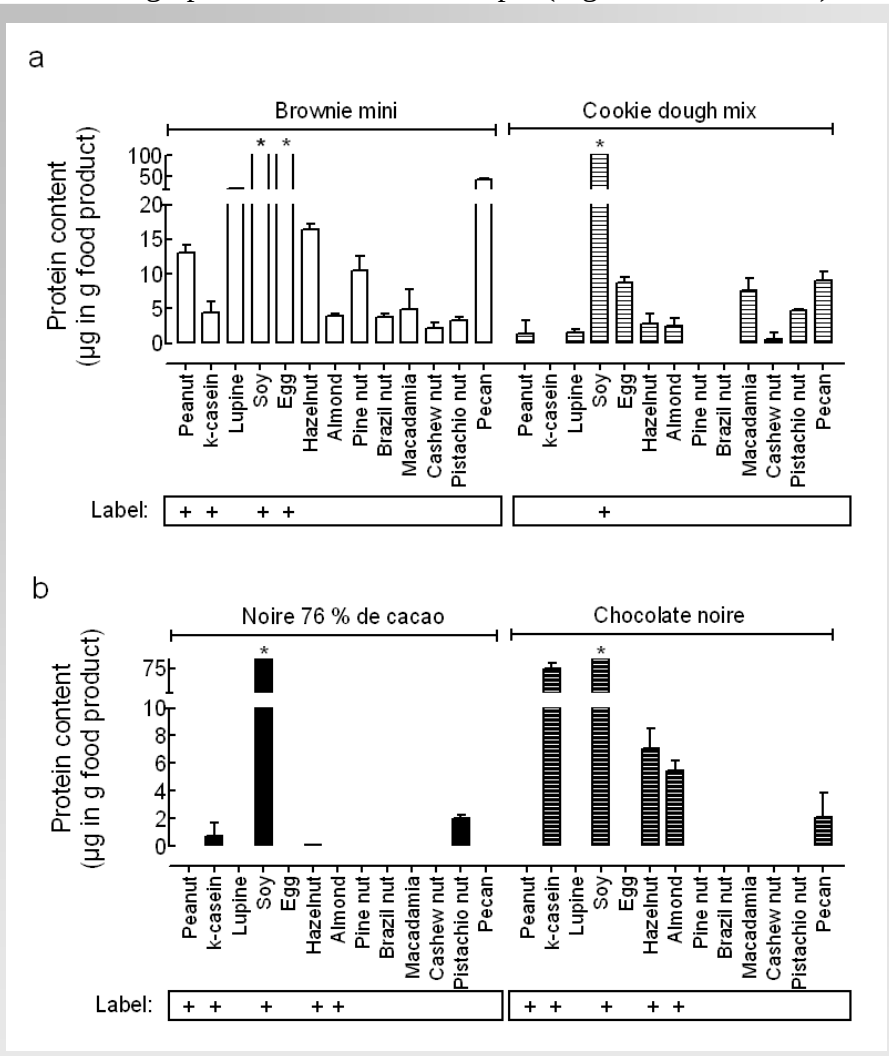


Figure 6.6 Allergen profiles of food samples. **(a)** Extracts of cookie dough mix (white) and brownie (white with stripes) were injected over the antibody micro-arrayed chip and maximal binding responses were measured on each spot at 480 seconds after sample injection. Asterisk indicates allergen concentration more than 100 µg per g food product. Error bars represent the standard deviations between duplicate spots and duplicate sample injections. **(b)** Allergenic profile of dark chocolates from two different manufacturers (black) and (black with stripes). Chocolate extracts were injected over an antibody-microarrayed chip and maximal binding responses were measured on each spot at 480 seconds after sample injection. Asterisk indicates allergen concentration more than 100 µg per g food product. Error bars represent the standard deviations (n=3).

For example, in brownie, higher peanut, milk, lupine, egg and hazelnut contents were found in comparison to cookie dough mix. Even though multiple nuts and egg were found in the cookie dough mix, no mentioning of these allergens was stated on the product label (Figure 6.6a and Table 6.2 in supplemental information). The analysis of two dark chocolates from different manufacturers showed differences in the milk, hazelnut and almond content. In this case, the contents of peanuts and milk were misrepresented on the

label (Figure 6.6b Table 6.2 in supplemental information). Usually dark chocolate sample extraction buffer includes milk powder which blocks binding of tannins to the allergens, but it also causes saturation on anti-κ-casein spots (Figure 6.6b and Table 6.2 in supplemental information**).** Thus bovine serum albumin or polyvinylpyrrolidone should be used instead of milk powder, during dark chocolate extraction when detecting milk traces (Figure 6.4 in supplemental information). High soy signals in dark chocolates and in cookies are caused by the presence of soy lecithin, commonly used as emulsifier in food products.

These examples demonstrate the power of direct allergen profiling on chip using iSPR. The obtained food profiles provide an extensive overview on the potential allergenicity of the food products, offering valuable information both for manufacturers and monitoring authorities. It is clear that this information is not readily accessible using currently employed single-analyte techniques, leading to false labelling, as presented above.

Conclusions

This study showed how direct on-chip allergen screening using iSPR can be applied to food profiling, offering a powerful analytical alternative to existing methods. Multiple allergen detection was achieved using on-chip direct iSPR-based analysis without labelling, signal amplification and washing steps, in a single reagent format. The obtained sensitivity was in the analytically relevant range and comparable to ELISA. Excellent applicability to allergens screening in food samples was demonstrated together with a broad detection spectrum and high robustness. With this approach, each food sample can be analysed within several minutes, faster than any other method currently available, providing a detailed and quantitative allergenic profile. High multiplexing capabilities and multiple measurements using a single chip contribute to reduction in the analysis costs. Furthermore, the automation of the iSPR system combined with sensor chip's stability presents a promising potential of implementation to in-line measurements, integrated into the manufacturing line. We believe that the method described here presents a cornerstone in food allergen analysis. It allows multi-analyte and high-throughput monitoring of food production equipment and food products and its routine application will contribute to correct product labelling, adequate legislation and -, foremost, safeguarding the health of allergic consumers.

Experimental Section

Antibodies generation

Production of anti- peanut (MAb 51-12D2), anti-hazelnut (MAb 51-12D2), anti-κ-casein (MAb 33-4G10) and anti-soy (MAb 3G12) was described previously 15,22,23. Polyclonal antibodies against lupine (PAb MH22) and egg (PAb MH7) were raised in rabbit according to the same immunization protocol as previously described for the development of anti-flumequine PAbs 24. Polyclonal antibodies against pine nut, almond, macadamia nut, brazil nut, cashew, pistachio and pecan were kindly provided by Dr. P. Delahaut, CER Groupe - Laboratoire d'Hormonologie, Belgium.

Food samples and allergen extraction

To 1 g of food sample (melted chocolate, ground cookie) 20 ml of pre-heated (60°C) RIDASCREEN® allergen extraction buffer (R-biopharm, Darmstadt, Germany) was added and mixed intensively. To reduce unspecific binding, 1 g of skimmed milk powder (MARVEL, UK) was added to chocolate samples. Extraction was done in 60 °C water bath with shaking for 10 minutes. The aqueous fraction was collected by centrifugation (3220 g for 10 minutes), and subsequently centrifuged again at 20,000 g for 10 minutes to remove residual fat and insoluble particles. The food extracts were divided into small aliquots (1 ml) and stored at -20 °C until used. For allergen extracts, 0.5 g of ground nut was diluted in 50 ml of PBS and incubated for 1 hour at room temp. Crude extracts were centrifuged at 3220 g for 20 minutes. The supernatant was collected and filtered through 0.45μm HT Tuffryn acrodisc syringe filter (Pall Life Sciences, UK). The protein content of all allergen extracts were determined using a BCA protein assay (PIERCE, Rockford, USA).

Antibody microarraying

Hydrogel coated sensor chip, pre-activated for amine coupling, (XanTec bioanalytics, Duesseldorf, Germany) was spotted with antibodies using continuous flow microfluidics spotter (Wasatch Microfluidics, Salt Lake City, USA). Optimal pH for each antibody immobilization was determined beforehand in surface preconcentration experiment using Biacore 3000. The spotter was washed with 0.1 % (w/v) SDS followed by 60 ^{o}C warm RO water, conditioned with 0.01 % (v/v) Tween 20 and primed with 5 mM acetic acid. The antibodies were prepared beforehand in 10 mM acetate buffer at pH and concentration as follows: anti-peanut pH 4.5, 0.005 mg/ml; anti-κ-casein pH 4.5, 0.005 mg/ml; anti-lupine pH 5, 0.01 mg/ml; anti-soy pH 4.5, 0.005 mg/ml; anti-hazelnut pH 4,

0.005 mg/ml; anti-almond pH 4.5, 0.01 mg/ml; anti-brazil nut pH 5, 0.005 mg/ml; anti-macadamia nut pH 5, 0.01 mg/ml; anti-cashew nut pH 4.5, 0.01 mg/ml; anti-pine nut pH 5, 0.01 mg/ml; anti-pistachio nut pH 5, 0.01 mg/ml; anti-pecan pH 5, 0.01 mg/ml. Each antibody was spotted in a duplicate in randomized manner over the sensor chip surface. During the spotting, six immobilization cycles were performed, each including 5 minutes contact time with the surface. Unreacted ester groups in the hydrogel were blocked with 0.5 M ethanolamine pH 8.5, for 10 minutes at RT. If not used immediately, the sensor chip was washed with RO water, dried under nitrogen stream and stored at 4 ^{o}C.

iSPR measurements

iSPR measurements were conducted using the IBIS iSPR instrument (IBIS Technologies B.V., Hengelo, The Netherlands). The sensor chip was assembled with the prism using refractive index matching oil in a round chip holder. A flow cell (4 µl volume) was fixed on top of the sensor chip surface. The sample was delivered to the sensor chip surface through a tubing and was pumped back and forth at 10 µl sec^{-1} during the interaction. The surface was equilibrated with the HBS-EP buffer (10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid pH 7.4, 150 mM sodium chloride, 3 mM EDTA, 0.005 % and (v/v) surfactant polysorbate (P20)) and regions of interest (ROIs) in size of 150 µm x 150 µm were defined on the spots. The SPR angle was scanned on each pre-defined ROI in the range between - 2.5 and + 2.8 degrees in steps of 200 millidegrees. SPR curves were fitted automatically by IBIS software while curve parameters were limited to 20 points before and after the dip. All the measurements were performed in the "analysis mode", recording SPR angle shift (m^{0}) as a function of time (sec). Subsequently, SPR data were analyzed using SPR inspection tool software ver. 1.6.0.0 (IBIS Technologies B.V. Hengelo, The Netherlands). Post measurement data sampling for each angle shift was done by averaging at least five data points collected around the desired time. Raw sensorgrams were first zeroed to the angle before the injection and then referenced to the angle of the blank spot. The maximum responses were calculated from the angle shift during the dissociation phase (around 600 seconds).

Allergens screening with iSPR

Thirteen direct immunoassays for allergens were multiplexed as follows. A freshly prepared sensor chip, microarrayed with antibodies directed against major food allergens, as described in the sensor chip preparation section, was conditioned with at least three serial injections (one minute contact time each) of regeneration solution containing 10 mM hydrochloric acid until the baselines of all the spots were stable in HBS-EP buffer. To test spot to spot

cross-contamination and antibodies cross-reactivity, each allergen at 5 ppm concentration was injected separately (7 minutes contact time) in duplicate over the surface of the pre-conditioned sensor chip. Next, multi-standard solutions containing all the allergens were prepared in HBS-EP buffer at concentrations ranging from 0.1 to 10 µg mL^{-1}. These allergen mixtures were injected over the sensor chip arrayed with the antibodies in duplicate, starting with blank solution containing only running buffer, blank cookie or chocolate. Each cycle included sample injection (7 minutes contact time) and one injection of regeneration solution (1 minutes contact time). All measurements were performed in duplicates and repeated at least on two different days on every chip. The maximal binding responses were also averaged between the spots containing the same antibody. To generate calibration curves, maximal responses were plotted against allergen concentrations for each antibody. The calibration curves were fitted with a non-linear one-phase model using GraphPad Prism software ver. 5.02 (GraphPad Software, Inc). The immunoassays were characterized by the limit of detection (LOD) and limit of quantitation (LOQ) which were calculated by adding three or ten, for LOD and for LOQ respectively, standard deviations to the average maximum response of blank cookie samples and blank dark chocolate samples. Food extracts measurements were done in the same way.

Hazelnut protein determination with ELISA

Hazelnut protein was quantified in the food samples, using sandwich ELISA (The RIDASCREEN® FAST kit, R-biopharm, Darmstadt, Germany). Food extracts were diluted using extraction buffer supplied with each kit. 150 µl of each standard solution and series of food sample extracts were first prepared in non-coated 96-well microtiter plate, and then 100 µl were transferred to antibody coated plate. The rest of the assay was performed according to the guidelines from the manufacturer. The absorbance was measured at 450 nm using ELx808 ultra Microplate Reader (BIO-TEK instruments, USA). Qualitative ELISA for hazelnut protein traces was performed as described previously [21].

Supplemental information

	R square	
	Non-linear [a]	Linear [b]
Peanut	0.9924	0.9838
κ-Casein	0.9944	0.9811
Lupine	0.994	0.9397
Egg	0.9827	0.8871
Soy	0.967	0.9274
Almond	0.9896	0.9288
Hazelnut	0.9986	0.9903
Pistachio nut	0.951	0.8661
Cashew nut	0.9855	0.8395
Macadamia	0.9781	0.853
Pecan	0.9795	0.9382
Brazil nut	0.9686	0.8127
Pine nut	0.9675	0.8767

Table 6.1 The goodness of fit with linear and non-linear models. **a-** Data was fitted with non-linear regression using pseudo-first order association kinetics between the antibodies and the allergens (concentration range 0-10ppm): Y=Y0 + (Plateau-Y0)*(1-exp(-K*x)). **b-**Data was fitted with linear regression (concentration range 0-2ppm): Y= YIntercept + Slope*X

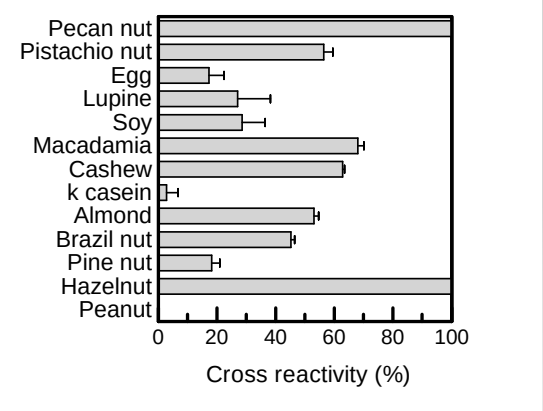


Figure 6.1 Cross-reactivity of anti-pecan towards different allergens used in this study. Standard solutions containing each allergen separately were prepared in HBS-EP buffer at concentrations of 5 µg mL^{-1} and injected over the antibody-microarrayed chip. Maximal binding responses were measured at 480 seconds after sample injection and compared to the maximal binding response of the specific antigen at the same concentration (100 %).

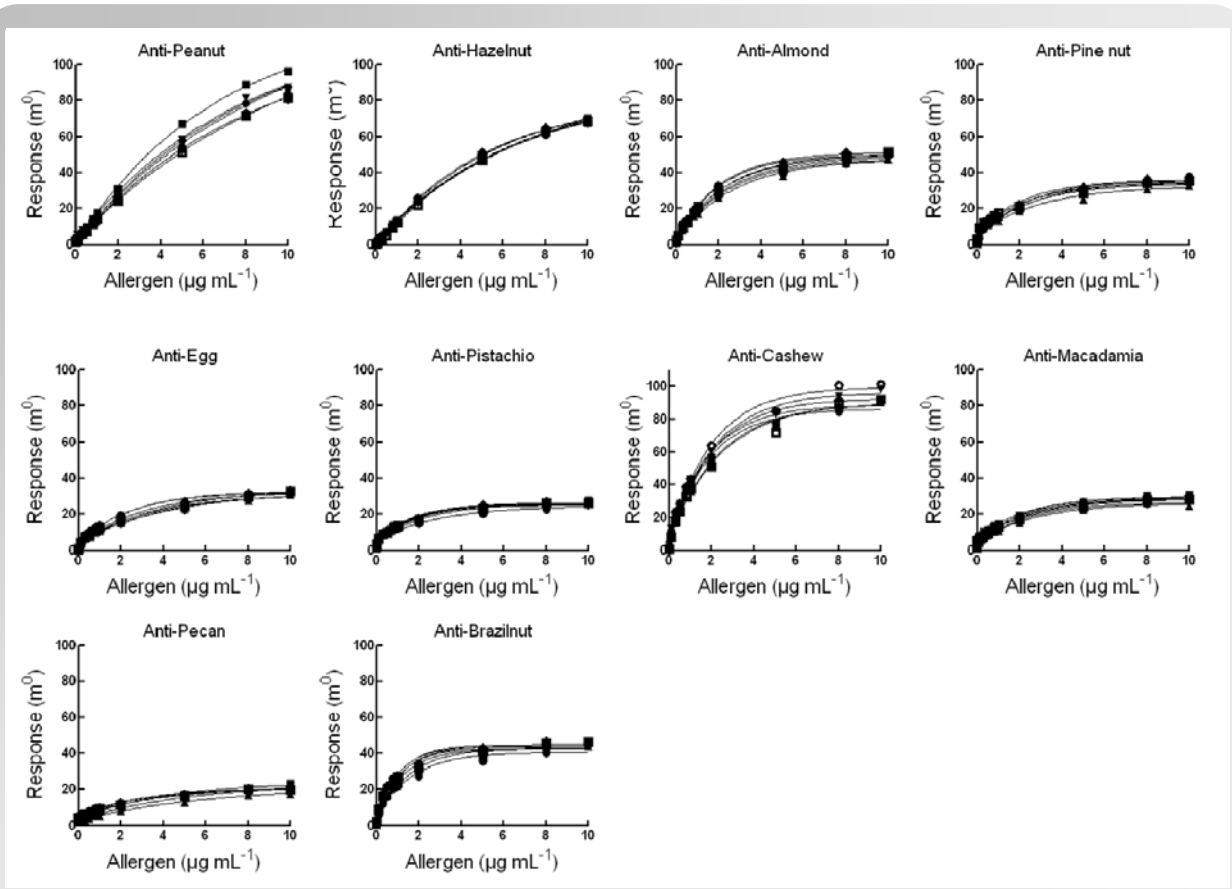


Figure 6.2 Stability of the rest of the antibodies used on the chip. Seven calibration curves, each including 20 injections, measured on anti-peanut, anti-almond, anti-pine nut, anti-egg, anti-pistachio, anti-cashew, anti-macadamia, anti-pecan and anti-brazil nut spots are shown. Solid lines show curves fitted with a non-linear one-phase association model.

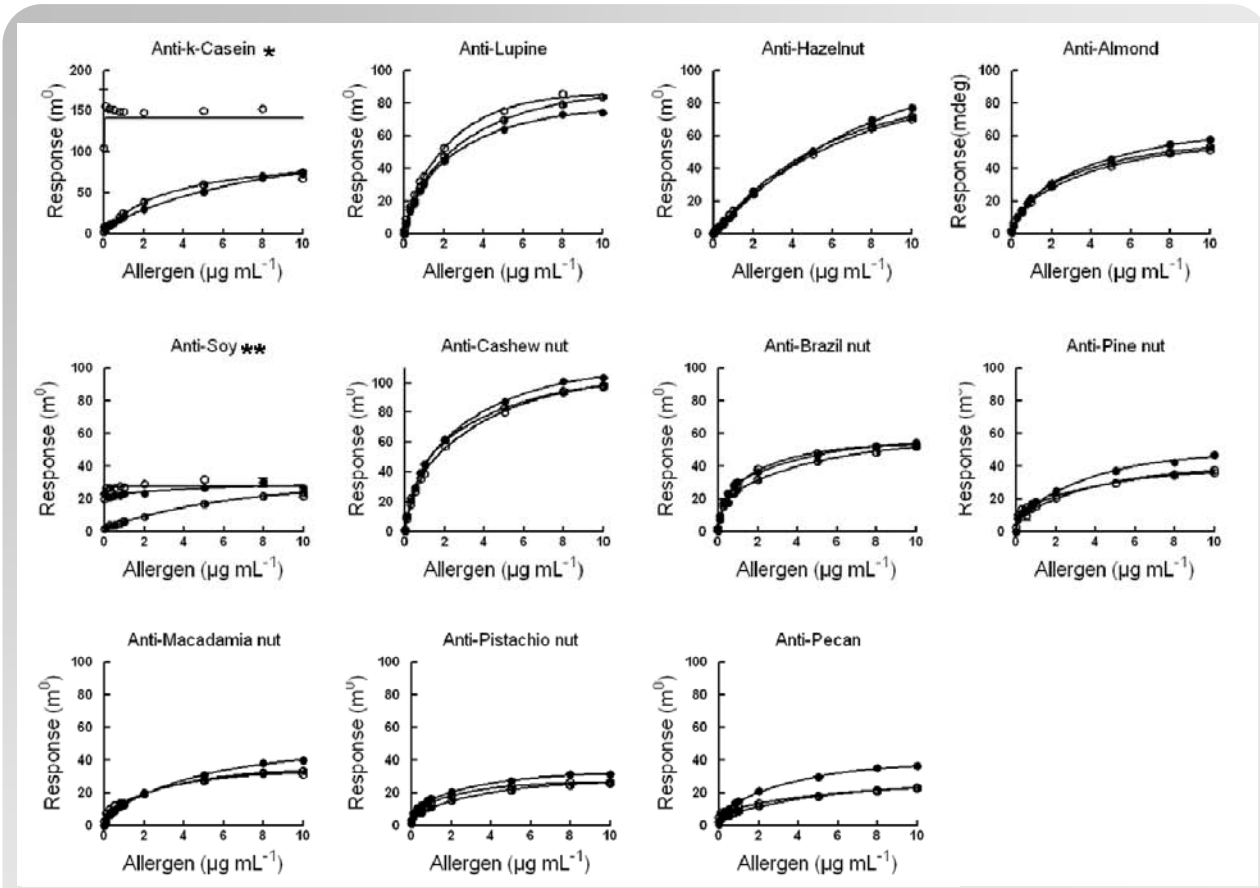


Figure 6.3 Food matrix effect on the rest of the antibodies used on the chip for the allergen measurements. Multi-analyte calibration curves in buffer (black and white), in cookie extract (black) and in dark chocolate extract (white). Solid lines show curves fitted with a non-linear one-phase association model. *- k-casein signals are saturated when skimmed milk is used for chocolate samples extraction. **- all commercial chocolates and cookies contain large amount of soy lecithin, thus the signals on soy antibody spots are saturated.

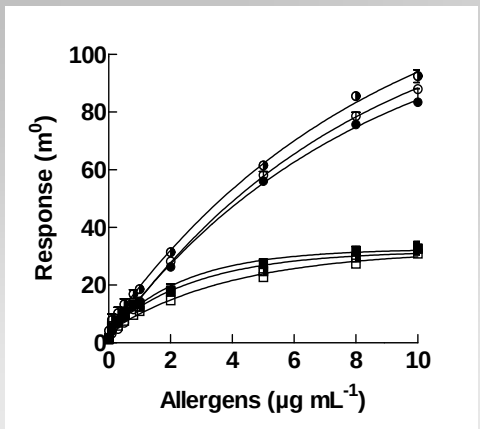


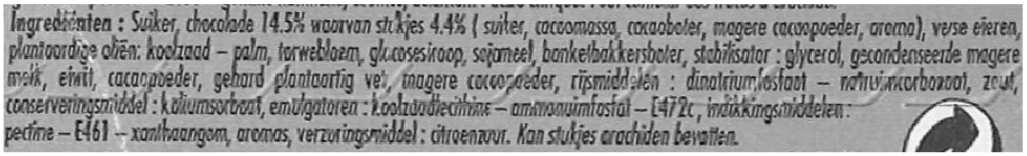
Figure 6.4 Dark chocolate effect on allergen measurements when milk or bovine serum albumin (BSA) are used for sample preparation. Multi-analyte calibration curves in buffer (white), in dark chocolate extract with milk (black and white) and in dark chocolate extract with BSA (black) measured on anti-egg (square) and on anti-peanut (circle) spots are shown. Solid lines show curves fitted with a non-linear one-phase association model.

Table 6.2 Food products ingredients as stated on the labels*.

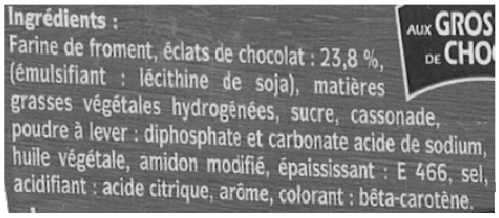
	Food product	Ingredients
C4	Brownie	Sugar, Chocolate, Fresh **eggs**, vegetable oil, palm oil, glucose syrup, **soy** flour, glycerol, concentrated skim **milk**, egg white, cocoa powder, rising and preserving agents. May contain **peanuts**.
C5	Mix for Cookie dough	Wheat flour, chocolate chips: 23.8% (emulsifier: **soy** lecithin), hydrogenated vegetable oil, brown sugar, rising and preserving agents, coloring: beta-carotene.
Ch2	Dark chocolate- "Chocolate Noire"	Cocoa mass, sugar, cocoa butter, vanilla flavoring, emulsifier: **soya** lecithin. Cocoa solids : 76% minimum. Traces of **nuts, peanuts, egg, gluten, milk** proteins.
Ch3	Dark chocolate- "Noire 76% de cacao"	Cocoa, sugar, cocoa butter, vanilla aroma, **soy** lecithin. Cocoa solids: 70% minimum. May contain traces of **peanuts, hazelnut, almonds and milk** solids.

*Original product labels:

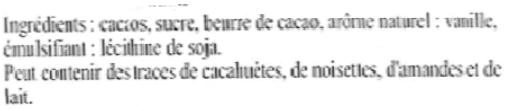
C4



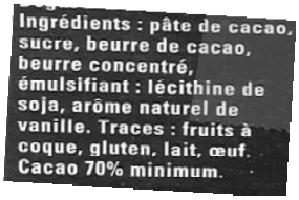
C5



Ch2



Ch3



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Chapter 7

Nanopatterned Submicron Pores on a Gel-supported Membrane for On-chip Sample Preparation in Surface Plasmon Resonance Sensing

This chapter describes a novel approach to tackle the most common drawback of using Surface Plasmon Resonance for analyte screening in complex biological matrices - the nonspecific binding to the sensor chip surface. By using a perforated membrane supported by a polymeric gel structure that exceeds the evanescent wave penetration depth, we created a filter above the sensing region. This filter prevents the diffusion of large particles or aggregates that bind nonspecifically to the polymeric gel and interfere with SPR sensing, and thus increases the assay's sensitivity, reduces sample preparation steps and shortens the analysis time in total. A non-fouling membrane with nanopatterned macropores was fabricated by means of colloidal lithography and plasma enhanced chemical vapor deposition of polyethylene oxide-like film. Such a membrane was supported by carboxymethyldextran, a polymeric gel matrix commonly used in surface plasmon resonance analysis. The surface was characterized using surface plasmon resonance imaging, contact angle, atomic force microscopy and scanning electron microscopy. The performance in full fat milk and porcine serum was studied using an immunoassay for detection of antibiotic as a model system. Structurally, the 92 ± 15 nm diameter pores presented an hexagonal crystal lattice and a clearance of about 5 % of the total surface. Functionally, the nanopatterned macropores showed significant improvements in the quality of the obtained measurements in comparison to bare carboxymethyldextran, displaying 100 fold decrease in enrofloxacin bioassay limit of detection when performed in porcine serum.

Submitted to Lab on a Chip.

Introduction

Surface Plasmon Resonance (SPR) sensing offers a powerful platform for molecular interactions monitoring, which capability has been abundantly demonstrated for a wide variety of applications [1-5]. In the analytical field, SPR-based biosensors are positioned as a rapid, quantitative, real-time and often automated technique. However, many SPR-based sensing analytical applications are hampered by the reduced performance in complex samples, which has been recently recognized as a severe limitation in the further development of the technology. Even though a separate sample pre-treatment is an option for some applications, the possibility to avoid it is one of the major advantages of SPR over traditional analytical methods and therefore has an important added value that should be preserved. The matrix interference with the bioassay performance is mainly attributed to nonspecific binding (NSB) of the sample components to the sensor chip surface [6, 7]. The inability to control NSB of several sample components, including lipids, lipoproteins and whole micelles, causes instable high background signals and poor repeatability of measurements in complex samples presenting a major technical challenge in bio-analytical assays development. The difficulties in bioassay transfer from optimal buffer conditions to real life samples are experienced by many researchers, compromising application possibilities of many bioassays and naturally leading to few publications that describe the problem. For example, at low analyte concentrations, the specific ligand-analyte interaction can be masked by the non-specific protein adsorption up to the point where the analyte is no longer detectable. This is the major difficulty in the development of SPR biosensors aimed towards medical monitoring, where the measurements are done in undiluted serum [8].

NSB essentially is a non-covalent binding, which is driven by hydrophobic, electrostatic and exchange interactions between molecules, usually attributed to protein adsorption [9, 10]. Since SPR is sensitive to a total refractive index change in close proximity to the sensor chip surface, there is no differentiation between the specific interaction that takes place between the ligand on the chip surface and the analyte in the sample, and non-specific interaction between the sample components and the sensor chip surface [7, 11, 12]. Most of the research has been directed towards reducing the non-specific binding either via introduction of new polymers as alternatives for commonly used carboxymethydextran (CM-dextran) or via sample treatment with NSB blocking additives [6-8, 13]. For example, Masson *et al*. looked at bioassay performance in full bovine serum using several biocompatible polymers on the SPR sensor chip. Even though some were found to be less prone to nonspecific binding, the CM-dextran still offered the largest signal for the antigen

detection [8]. In a different study by Elliot *et al.*, a buffering system, supplemented with soluble CM-dextran, was reported to reduce the nonspecific interactions of bovine serum components with the sensor chip. The most substantial reduction achieved with this approach still required 10 fold dilution of the sample. Moreover, even under the optimized conditions, the total binding signal in the matrix was lower and the IC_{50} values were higher in 10 fold diluted bovine serum than in buffer [6].

Here, we propose an alternative approach to reduce NSB binding by on-chip sample preparation. Taking advantage of the limited Surface Plasmon Waves penetration depth (usually around 300 nm), 500 nm thick CM-dextran was utilized as a support platform for a submicron porous material in order to create a filtering layer on top of the sensing region. Ligands were covalently immobilized in the CM-dextran layer, where the specific interaction with the analyte occurred, while insoluble aggregates and other large matrix components were filtered out by the filtering layer of PEO. The filtering layer was constructed out of non-fouling membrane (less than 10 nm thick) with nanopatterned macropores which was fabricated by means of colloidal lithography and plasma enhanced vapor deposition of polyethylene oxide on top of CM-dextran (PEO-pores)[14]. Many nanoporous materials are used in biological applications, however submicron nanopatterned polyethylene oxide-based membrane have not been reported before [15]. The influence of PEO-pores sensor chip coverage on biomolecular interactions in buffer and in complex samples was studied using imaging SPR in direct comparison to commonly used CM-dextran. As a model for analytical application, a previously described SPR-based immunoassay for enrofloxacin detection was used [16]. For complex samples, with target analyte pertinence, full fat milk and porcine serum were chosen. Full fat milk contains high amounts of protein as well as fat and casein micelles which seem to interfere with the SPR measurements [17]. Even though the specific factors causing NSB in porcine serum are presently unidentified, it is also considered to be a difficult matrix for analysis with many analytical methods including SPR biosensors.

Results and Discussion

Nanofabrication and biomolecular interactions on PEO-pores

One half of the carboxymethylated dextran chip was covered with non-fouling PEO membrane containing nanopatterned macropores (PEO-pores), leaving the other half of the surface untreated with PEO as a control (CM-dextran). The diagram in Figure 7.1 shows the nanofabrication process of the

pores in PEO membrane on top of the carboxymethyldextran sensor chip. The dextran surface was spin coated with a polystyrene nano-sphere mask achieving approximately 65 % coverage. The other half of the chip was masked to include a control area without PEO-pores; then, a PEO layer was plasma deposited on top of the bead nanomask. Upon nanomask liftoff, an array of macropores with an hexagonal lattice and a diameter of 92 ± 15 nm was created on the PEO layer as shown by atomic force microscopy (AFM) (Figure 7.1, bottom right) and scanning electron microscopy (SEM) (Figure7.1, bottom right). The area of the CM-dextran, which was masked to prevent PEO deposition, showed superficial random roughness in AFM (Figure 7.1, bottom left) and a significantly higher hydrophilicity (contact angle 25.5 ± 0.9°), when compared to PEO-pores (contact angle 52 ± 1.8°).

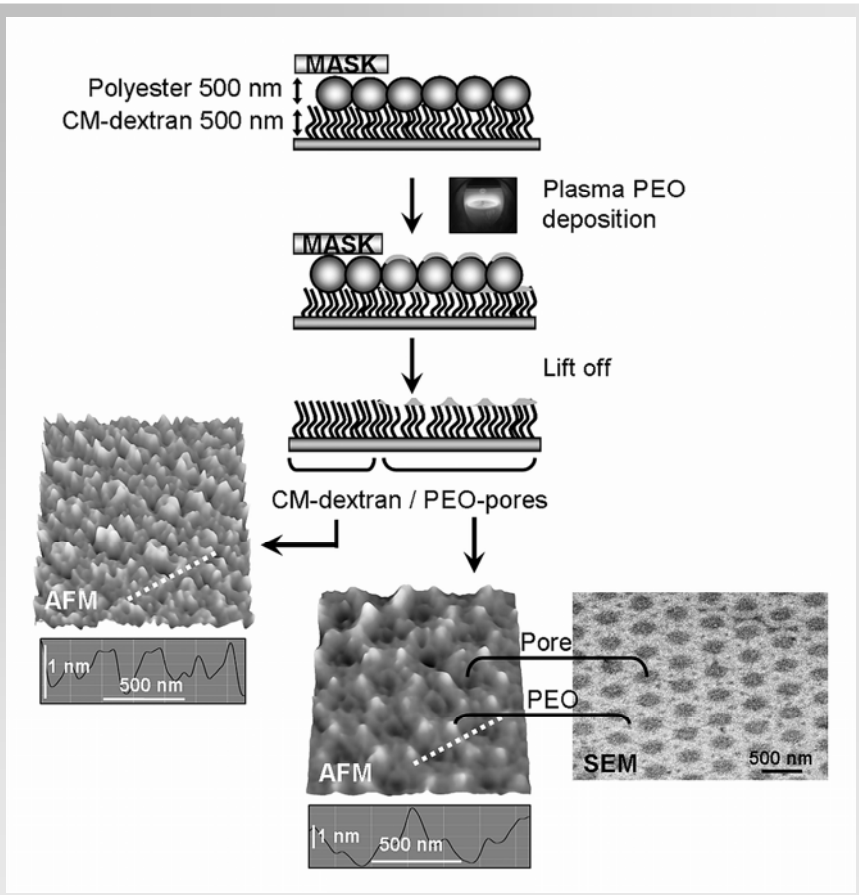

Figure 7.1 Sensor chip nanofabrication process and characterization. SPR sensor chip is spin-coated with 500 nm polystyrene beads. Then, half of the sensor chip surface is masked and PEO is plasma deposited, producing a PEO layer (up to10 nm thick) on top of the CM-dextran. After the bead scaffold liftoff, the nanofabricated sensor chip surface is characterized by means of atomic force microscopy (AFM) and scanning electron microscopy (SEM). The sensor chip surface with plasma deposited PEO (PEO-pores) shows a clear hexagonal lattice pattern (bottom right, AFM and SEM images), as opposed to the surface which was masked (CM-dextran) (bottom left, AFM image).

To study PEO-pores compatibility with applications in biomolecular interaction analysis, two main points were addressed: surface capacity to ligand immobilization and analyte binding to the ligand. High and low molecular weight compounds, anti-κ-casein antibody (αCas) and norfloxacin antibiotic (NorF), were covalently immobilized on both

halves of the sensor chip (CM-dextran and PEO-pores) using a continuous flow microfluidics spotter (CFM) (Figure 7.2A and 7.2B). The iSPR image of the spotted sensor chip shows slightly brighter regions in the lower part of the sensor chip close to the mask boundaries (see Figure 7.2C, marked with an arrow), indicating a certain level of isotropy in the plasma deposition of PEO. Spots 4, 5 and 6 appear brighter than the rest, corresponding to higher angle shift due to higher molecular weight ligand (αCas) immobilization. As expected, on the rest of the spots the immobilization is not evident from the SPR image, due to the low molecular weight of NorF (Figure 7.2C). To be able to monitor the binding reaction on NorF spots, the grid of regions of interest (ROIs) was placed accordingly to αCas spots.

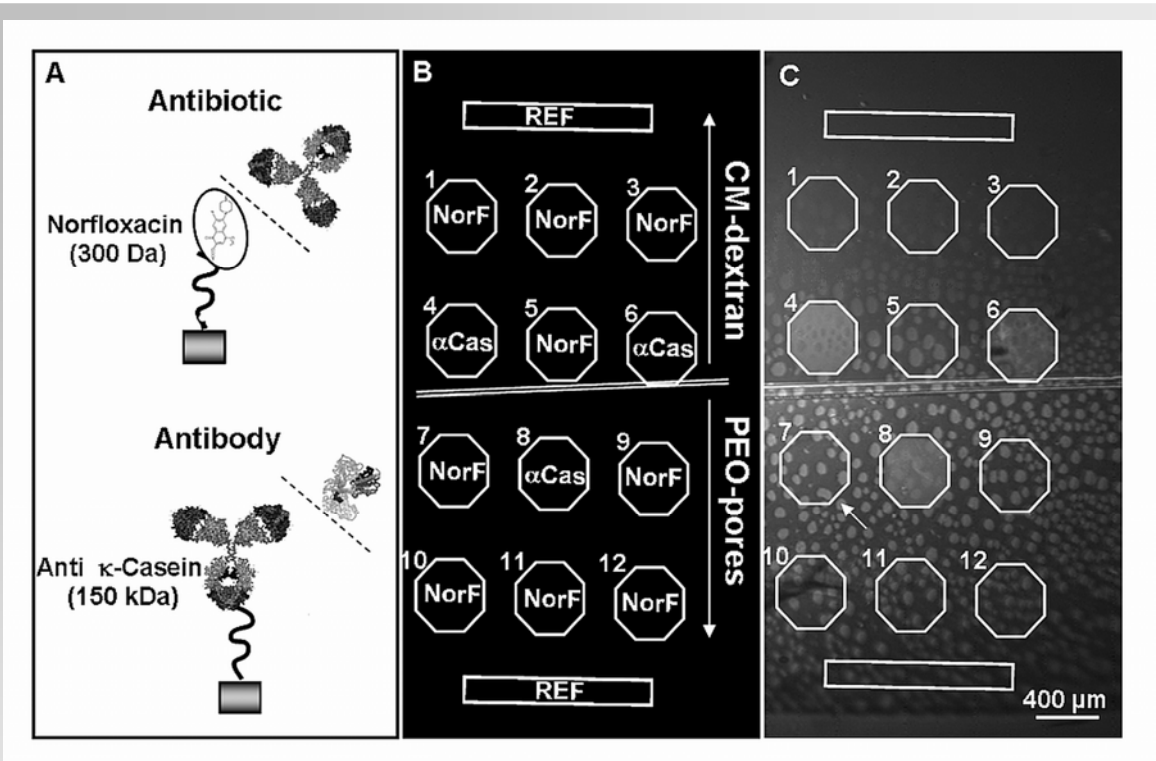

Figure 7.2 Sensor chip lay out. A- Schematic representation (out of scale) of immobilized ligands on the sensor chip. Both high molecular weight (antibody) and low (antibiotics) molecular weight ligands were covalently immobilized, via primary amines, on the PEO-pores and on CM-dextran simultaneously using continuous flow spotter (CFM). The immobilization was tested with injections of anti-Norfloxacin antibody and k-casein. B- Spotting lay out for CM-dextran and for PEO-pores, including Norfloxacin – NH_2 spots (NorF), anti-casein antibody spots (αCas) and blank spots which were used for signal referencing in imaging Surface Plasmon Resonance measurements. The octagonals show the spotted regions. On spots number 4, 6 and 8 anti-casein antibody was immobilized and on the rest Norfloxacin – NH_2. The double line in the middle shows the applied mask border during the PEO-pores production. C- iSPR image of the spotted sensor chip. Arrow indicates brighter regions with isotropic PEO deposition.

To test the immobilization efficiency, κ-casein and anti-enrofloxacin antibody were serially injected over the spotted surfaces. Maximal binding responses measured on αCas spots were comparable for PEO-pores and CM-dextran, showing both good ability for

high molecular weight ligand immobilization and penetration (Figure 7.3A). Maximal binding responses measured on NorF spots were 30 % lower on PEO-pores than on CM-dextran surface, indicating either lower ligand immobilization level or slower diffusion of the high molecular weight analyte (Figure 7.3A). Prolonged injection of higher diluted antibody showed a faster surface saturation rate on PEO-pores than on CM-dextran surface, indicating slightly lower immobilization levels of norfloxacin on PEO-pores (Figure 7.3B). The differences between PEO-pores capacity for anti-casein antibody and norfloxacin antibiotic might be attributed to different hydrophobicity of the two ligands. Norfloxacin, even though is a small molecule and is expected to migrate easily through the PEO-pores, is quite hydrophobic and might prefer to pre-concentrate on the less hydrophilic PEO membrane, instead of diffusing further to the hydrophilic CM-dextran, where the immobilization occurs. Overall, although the PEO-pores reduce in about 95 % the exposed CM-dextran area, they show no substantial interference neither with the immobilization of large and small molecules on the sensor chip surface, nor with ligand-analyte binding, thus suggesting a good compatibility with biomolecular interaction analysis.

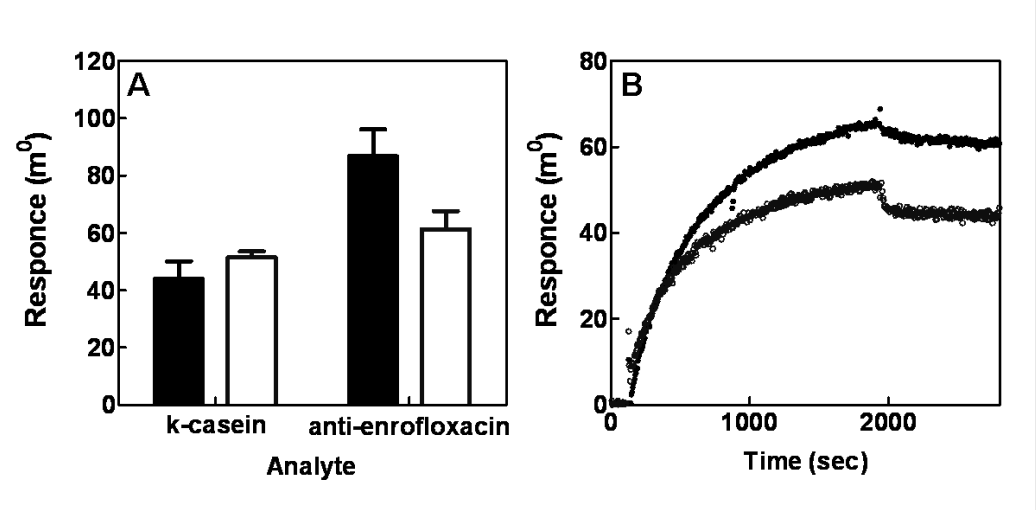

Figure 7.3 Biomolecular interactions on PEO-pores. A- Maximal binding responses measured on anti-casein and NorF spots, both on PEO-pores (hollow blue) and on CM-dextran (black) surfaces, after serial injections (7 minutes each) of κ-casein (5 μg mL^{-1}) and anti enrofloxacin antibody (1:200). Error bars show standard deviations between the spots during triplicate injections. B- Representative blanked and referenced sensorgrams measured on NorF/PEO-pores (blue) and NorF/CM-dextran (black) spots during prolonged injection of anti-enrofloxacin antibody (1:400), showing surface saturation rates.

Concentration measurements in complex matrices on PEO-pores

To study the performance of PEO-pores in complex matrices, an immunoassay for enrofloxacin detection was performed in a full fat milk and porcine serum. The assay was formerly established for the detection of fluoroquinolones using SPR biosensing, but

showed a lower performance in chicken serum [16]. Here, full fat milk and porcine serum were only subjected to dilution in a running buffer, without additional filtering and centrifugation steps, which are usually applied in such cases [17, 18]. The immunoassay was performed in the competitive format, based on inhibition of antibody - binding to the antibiotic immobilized on the surface by the antibiotic in solution. Enrofloxacin standard solutions were prepared in buffer, milk and serum at different concentrations, mixed with the anti-enrofloxacin antibody and injected over norfloxacin spotted sensor chip, covered with PEO-pores (upper half) and with CM-dextran (lower half).The antibody for enrofloxacin used in this study is known to cross-react with norfloxacin, which was used in this case for immobilization on chip [16]. Figure 7.4A shows four raw sensorgrams measured in porcine serum, during duplicate injections of anti-enrofloxacin antibody without the presence of antibiotics, on the NorF/PEO-pores (blue) and on the NorF/CM-dextran (black) spots. Each single measurement cycle included surface equilibration with buffer, sample injection, dissociation in buffer, regeneration and re-equilibration with buffer. Sensorgrams on PEO-pores showed lower binding responses than on CM-dextran, as expected, due to lower norfloxacin immobilization levels. Raw sensorgrams measured on NorF/PEO-pores and on NorF/CM-dextran spots were zeroed to the buffer baseline before the sample injection and referenced to REF/PEO-pores and to REF/CM-dextran spots, respectively. Spots 9, 11 and 12 on PEO-pores and spots 1,2 and 3 on CM-dextran were taken for analysis (Figure 7.2). Figure 7.4B shows representative zeroed and referenced sensorgrams measured on NorF/PEO-pores (blue) and on the NorF/CM-dextran (black) spots during injections of increasing enrofloxacin concentrations in serum. Even though the binding responses on CM-dextran are higher, the binding is not completely inhibited by high enrofloxacin concentrations, indicating non-specific binding to CM-dextran, as opposite to PEO-pores. For each surface, calibration curves in buffer, in milk and serum were plotted using relative binding values (B/B_0) as a function of enrofloxacin concentration (ng mL^{-1}) (Figure 7.4C, 7.4D, 7.4E). The curves were fitted with the 4-parameter non-linear model and limit of detection (LOD) was calculated (Figure 7.4C, 7.4D, 7.4E insets). For comparison, same experiment was performed on a standard 200 nm CM-dextran sensor chip, which is usually used for this kind of assays (grey triangle in Figure 7.4C, 7.4D, 7.4E). The calibration curves measured on PEO-pores and CM-dextran in buffer completely overlap, and display very similar LOD, IC_{50} and dynamic range. In milk and serum, the calibration curves measured on CM-dextran reach 80 % inhibition plateau around 80 pg mL^{-1} enrofloxacin, showing no response to further

increasing concentrations. On the contrary, the calibration curves measured on PEO-pores show full signal inhibition in milk at 2000 pg mL^{-1} enrofloxacin, and 95 % inhibition at 10000 pg mL^{-1} enrofloxacin (Figure 7.4C, 7.4D, 7.4E).

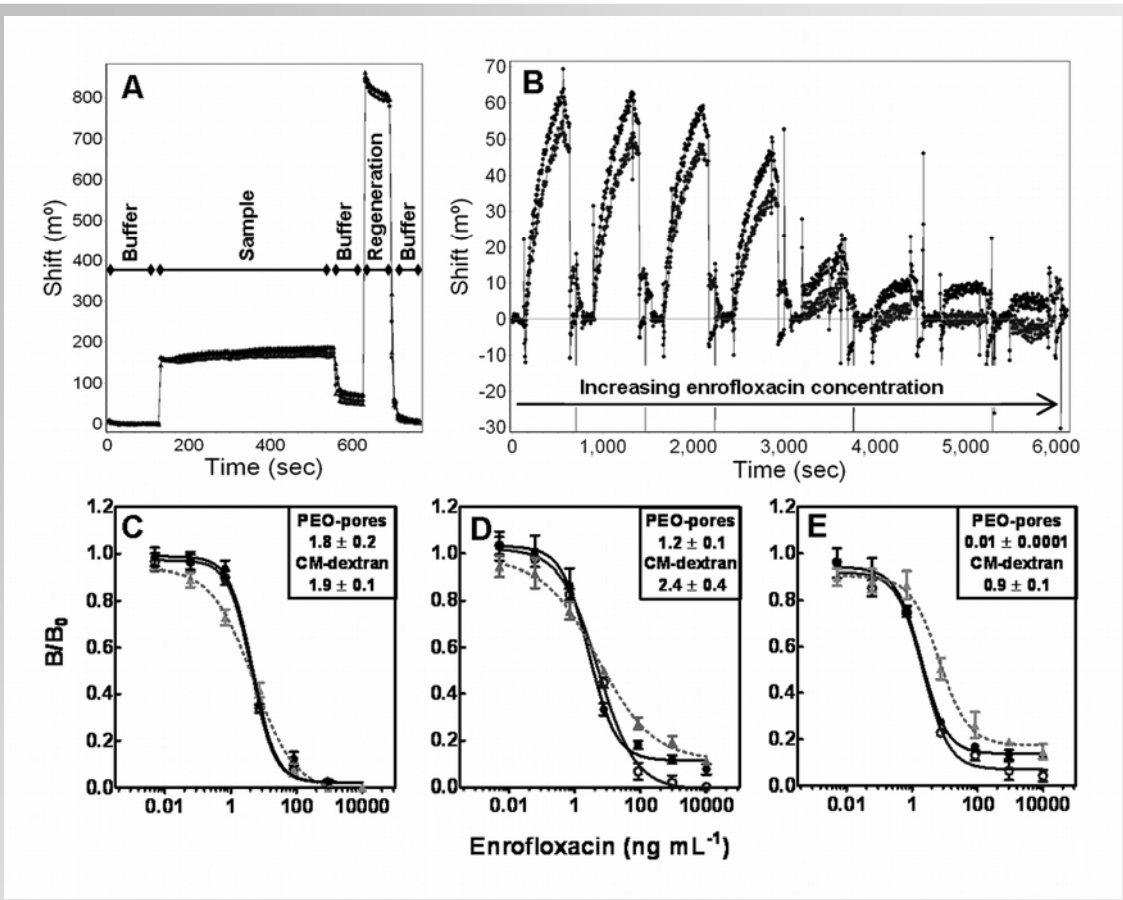

Figure 7.4 Concentration measurements in complex matrices on PEO-pores. A- Raw sensorgram measured on NorF/PEO-pores (hollow blue) and NorF/CM-dextran (black) spots during duplicate injections of anti-enrofloxacin antibody in diluted porcine serum. Each measurement cycle lasted 13 minutes, including 7 minutes incubation with the sample and 2 minutes regeneration with 20% ACN in 10mM NaOH. Maximum binding responses were calculated from the angle shift during the dissociation phase (around 600 seconds), after zeroing and referencing to a blank spot. B- Zeroed and referenced sensorgrams measured on NorF/PEO-pores (hollow blue) and NorF/CM-dextran (black) spots during injections of anti-enrofloxacin antibody mixed with enrofloxacin spiked into a diluted porcine serum at 0, 0.005, 0.06, 0.675, 7.5, 82.6, 909 and 10000 ng mL^{-1} final concentrations. C, D and E – Enrofloxacin calibration curves measured in buffer, diluted full fat milk and diluted porcine serum respectively, On NorF/PEO-pores (blue) and NorF/CM-dextran (black) and on separate 200 nm CM-dextran sensor chip (grey). B/B_0 stands for relative binding, calculated from the maximum binding response of the sample containing antibiotics (B) and the maximum binding response of the sample containing only the antibody (B_0). Insets indicate the limits of detection calculated for each matrix. The error bars represent standard deviations between three measurements performed in duplicate on different days. The lines show curves fitted with non-linear 4-parameters variable slope model.

Calibration curves that do not reach full inhibition at the highest analyte concentrations usually suggest unspecific binding, since the analyte is not able to fully suppress the signal. This phenomenon is commonly observed in complex matrices, while the degree of interference varies between the assays. When the unspecific binding causes the plateau at

less than 70 % inhibition, the assays are usually considered to be not applicable to measurements in real life samples (unpublished data). Additionally, assay's robustness presents a challenge in complex matrices. Often, the variation between independent measurements is too high in complex matrix to produce reliable results. PEO-pores showed lower standard deviations, both in milk (10 % on PEO-pores ver. 15 % on CM-dextran) and in serum (2 % on PEO-pores ver. 10% on CM-dextran) than the CM-dextran, thus improving both assay's robustness and hence the limit of detection (LOD) (Figure 7.4D and 7.4E), providing better controlled conditions for the bioassay performance. Same trend was observed in comparison to 200 nm CM-dextran. Reducing NSB by PEO-pores is also evident from the iSPR measurements in milk and serum on blank (REF/CM-dextran and REF/PEO-pores) spots (Figure 7.5). Figure 7.5A shows zeroed sensorgrams measured on the REF/PEO-pores and REF/CM-dextran during buffer, milk and serum injections. Bulk responses, non-specific binding and baseline build up were calculated from the angle shift at 550, 600 and 750 seconds, respectively. While the bulk responses did not differ much on CM-dextran and PEO-surfaces, the NSB was evidently lower on PEO-pores: 50 % reduction in milk and 25 % reduction in serum (Figure 7.5B and 7.5C). This figure also shows that non-specific binding is the highest in porcine serum, for both surfaces, which agrees with the inhibition plateau observed with enrofloxacin assay in serum. Additionally, an interesting trend in baseline build up was observed (Figure 7.5D). PEO-pores showed rather constant baseline build up in each measurement cycle, whereas CM-dextran baseline build up was somewhat random in serum and milk (between 0 and 10 m^{o}). This suggests unsystematic interactions between matrix components and the sensor chip surface on CM-dextran, contrarily to PEO-pores, which might explain higher variation in binding responses and the associated larger standard deviations observed with the enrofloxacin assay. The improvement with using PEO-pores in milk may be attributed to keeping the fat and caseins micelles outside of the sensing region. However, to be able to fully explain the mechanism behind the PEO-pores functionality first the origin of unspecific binding in porcine serum should be studied. Overall PEO-pores displayed a promising ability to reduce negative matrix effects, improving assay's sensitivity at both high and low analyte concentrations. Significant 100-fold improvement of the LOD in serum could mean the difference between a theoretically applicable and actually working assay. The ability to improve the assay's sensitivity in complex matrices by simultaneous on-chip sample preparation also compensates for the reduction in sensitivity when comparing to immunoassays in the traditional format such as ELISA.

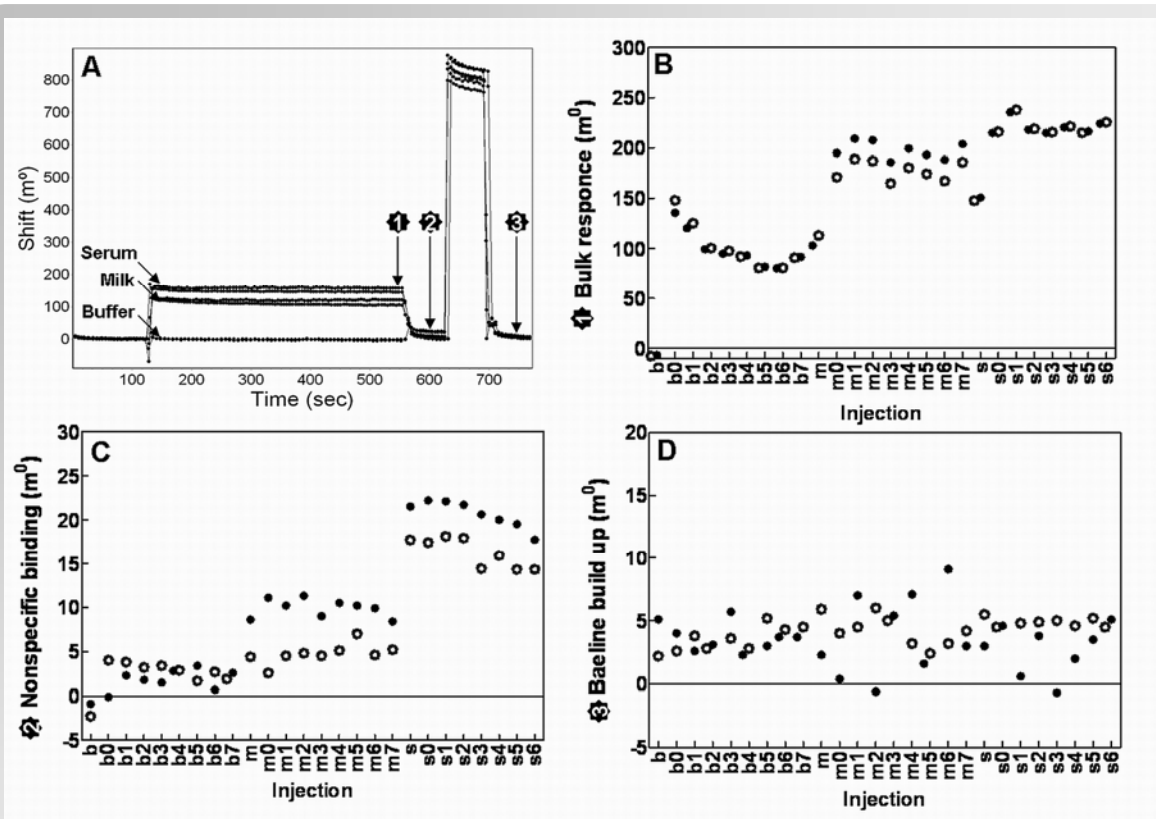

Figure 7.5 iSPR measurements on blank PEO-pores in complex matrices. A- Zeroed sensorgrams measured on blank PEO-pores (hollow blue) and blank CM-dextran (black) during buffer, diluted full fat milk and diluted porcine serum injections. B, C and D- Bulk responses, non-specific binding and baseline build up, respectively, on blank PEO-pores (hollow blue) and blank CM-dextran (black) measured during enrofloxacin calibration curve injections in buffer, diluted full fat milk and diluted porcine serum. For bulk response calculation, the angle shift before the end of sample injection (1) was taken (around 550 seconds). For non-specific binding calculation, the angle shift during the dissociation phase (2) was taken (around 600 seconds). For baseline build up calculation, the angle shift after the regeneration (3) was taken (around 750 seconds).

Conclusions

Nonspecific binding of sample components in complex matrices to the sensor surface is a common bottleneck in the development of analytical bioassays on SPR platform, usually tackled through extensive sample pretreatment. By engineering the sensor chip surface, we proposed a simultaneous sample pretreatment and analysis. Nanopatterned PEO membrane (PEO-pores), on top of 500 nm high carboxymethylated dextran layer, enabled filtering out large particles in the sample matrix preventing non specific binding to the sensor chip surface in the SPW probed field. PEO-pores displayed lower hydrophylicity than dextran, but did not compromise neither immobilization nor molecular interaction of high and low molecular weight compounds. Utilization of the PEO-pores for antibiotic detection bioassay in full fat milk and porcine serum resulted in reduced non-specific binding, higher assay robustness and almost 100-fold reduction in LOD. The source of NSB is matrix dependent, and its source is

often not identified. Here we applied a general approach to reduce the NSB by filtering; however, in principle, the surface can be also similarly engineered to match specific application needs. Following the proof of principle, presented here, future work will focus on further exploration of different PEO-pores sizes and their performance in different complex matrices along with more detailed characterization of different sources for NSB. In our view, the advances described for on-chip sample preparation show a great potential to improve not only SPR-based assays performance in complex matrices but also other optical evanescent wave-based sensor devices suffering from similar sample preparation drawback. Thus, the proposed method widens the application range of these technologies in the bioanalytical field.

Experimental Section

Chemicals and Materials

Round sensor chips, coated with 500 nm or 200nm high carboxymethyldextran with a medium degree of cross-linkage, were purchased from Xantec bioanalytics GmbH (Duesseldorf, Germany). Biacore amine coupling kit (containing 0.1 M N-hydroxysuccinimide (NHS), 0.4 M N-ethyl-N-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1 M ethanolamine hydrochloride pH 8.5 HBS-EP buffer) were purchased from GE Healthcare (Uppsala, Sweden). Norfloxacin-NH_2 derivative (NorF) was kindly supplied by Dr. Sheryl Tittlemier (Health Canada, Ottawa). Rabbit polyclonal antiserum (MAR06101), raised against a norfloxacin-COOH derivative was kindly supplied by Laboratoire d'Hormonologie Animale (Marloie, Belgium). Monoclonal anti-κ-casein (Mab 4G10) antibody was previously described elsewhere [19], [19]. Porcine serum was kindly provided by Central Veterinary Institute (Lelystad, Netherlands). The full-fat goats' milk powder (Mekkermelk from Henri Willig, Katwoude, The Netherlands) was purchased locally. The iSPR instrument, round sensor chip holder, refractive index matching oil (n=1.518), hemispheric prism (BK7) and a 20 µl flow cell were purchased from IBIS Technologies B.V. (Hengelo, The Netherlands). Diethylene glycol dimethyl ether (DEGDME) $(CH_3OCH_2CH_2)_2O$, polystyrene beads (500 nm diameter, 10 % monodispersity) and the rest of the chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Colloidal lithography

The PEO-pores were produced by nano-sphere lithography. Briefly, a polystyrene bead monolayer

nano-mask was deposited as described elsewhere [20], by spin-coating a bead solution on a 500 nm-thick dry carboxymethylated dextran layer covering gold sensor chips.

Plasma deposition of the polyethylene oxide layer

The deposition of a 10 nm thick film of plasma polymerized polyethylene oxide (PEO) was performed in a homemade stainless-steel (300 mm × 300 mm × 150 mm) reactor with two symmetrical internal parallel-plate electrodes (diameter of electrodes = 140 mm, distance between the two electrodes = 50 mm). The plasma was generated by a radio frequency generator (13.56 MHz) connected to the upper electrode whereas the other electrode was grounded and used as a sample holder. The plasma polymerization was carried out by using a pulsed plasma discharge (time on = 10 ms, time off = 100 ms, nominal power = 5 W) of DEGDME vapors. Further description of the reactor can be found elsewhere [21]. For the nanomask lift-off, after the plasma PEO deposition, the sensor chips were submerged in distilled water and sonicated for five minutes.

Atomic Force Microscopy (AFM)

Nanofabricated and control sensor chip surfaces were imaged with an Agilent AFM 5500 microscope (Agilent Tecnologies Inc. - Santa Clara, California, U.S.A.) under a standard setup, in Magnetic AC mode, using MAClever type V silicon nitride tips (spring constant = 0.5 N/m).

Scanning Electron Microscopy (SEM)

The PEO-pores on the dextran chips were imaged using SEM FEI Nova 600I Nanolab (FEI , Eindhoven, The Netherlands) at relatively low acceleration voltages (5KV) that allow the use of an immersion lens detector without significant loss of resolution. Samples were grounded contacting the conductive gold layer to the ground of the microscope to avoid charging effects on the images.

Ligands microarraying

The sensor chip, partly covered by PEO-pores, was activated offline with the EDC/NHS (1:1) mixture, rinsed with 5mM acetic acid solution, dried under nitrogen stream and immediately spotted with ligands using the continuous flow microfluidic (CFM) spotter (Wasatch Microfluidics, Salt Lake City, USA). The ligands were prepared beforehand as follows. NorF was dissolved in 20 mM carbonate buffer pH 8.5 with 30 % (v/v) dimethylformamide (DMF) and immobilized in 10 mM carbonate buffer pH 9.6 at a final concentration of 0.3 mM. Anti-k-casein antibody was Protein G purified, desalted in to 20 mM acetate buffer pH 4.5 and immobilized at a final concentration of 0.05 μg mL^{-1}. During the spotting, 6

immobilization cycles were applied, 5 minutes each. 20 mM acetate buffer pH 4.5 was used as a priming buffer during the spotting. Unreacted groups in the CM-dextran were blocked with 0.5 M ethanolamine pH 8.5, for 10 minutes. If not used, the sensor chip was washed with RO water, dried under nitrogen stream and stored at 4 ^{o}C.

SPR measurements

iSPR measurements were conducted using the IBIS iSPR instrument. The sensor chip was assembled with the prism using refractive index matching oil in a round chip holder and the flow cell was fixed on top of the sensor chip surface. The sample was delivered to the sensor chip surface through a tubing and was pumped back and forth at 10 µl sec^{-1} during the interaction (7 min). The surface was equilibrated with the HBS-EP buffer (containing 10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid pH 7.4, 150 mM sodium chloride, 3 mM EDTA, 0.005% and (v/v) surfactant polysorbate (P20) and regions of interest (ROIs) in size of 300 µm x 300 µm were defined on the spots. The SPR angle was scanned on each pre-defined ROI in the range between – 3^{o} and + 1^{o} in steps of 200 m^{o} (one data point every 5 seconds). SPR curves were fitted automatically by IBIS software ver. 4.5 while curve parameters were limited to 20 points before and after the dip. All the measurements were performed in the "analysis mode", recording SPR angle shift (m^{o}) as a function of time (sec). Subsequently, SPR data were analyzed using SPR inspection tool software ver. 1.6.0.0 (IBIS Technologies B.V., Hengelo, The Netherlands). Post-measurement data sampling for each angle shift was done by averaging at least five data points collected around the desired time. Raw sensorgrams were first zeroed to the angle before the injection and then referenced to the angle of the blank spot (REF), either on PEO-pores or on CM-dextran. The maximum binding responses on NorF or αCas spots and non-specific binding on REF spots were calculated from the angle shift during the dissociation phase (around 600 seconds). Bulk responses and baseline build up on REF spots, were calculated from the angle shift before the end of sample injection (around 550 seconds) and the angle shift after the regeneration (around 750 seconds), respectively.

Immobilization check and concentration measurements

A freshly prepared sensor chip, partly covered with PEO-pores and microarrayed with NorF and αCas, was first tested for the ligands immobilization efficiency. Since the regeneration solution for the interaction of norfloxacin and anti-enrofloxacin antibody causes loss of anti-k-casein antibody activity, the immobilization of αCas was checked first. The surface was conditioned with at least

three serial injections (two minutes contact time each) of regeneration solution containing 10 mM HCl until the baselines of αCas spots were stable in HBS-EP buffer. κ-casein was injected at a final concentration of 5 $\mu g mL^{-1}$ in HBS-EP buffer and the maximal binding responses on αCas were measured. Same was repeated with anti-enrofloxacin (1:200) in HBS-EP on NorF spots, only under harsher regeneration conditions: 20% (v/v) ACN in 10 mM NaOH. Surfaces saturation rate was tested by a longer injection of anti-enrofloxacin (1:400) in HBS-EP on NorF spots. From theses steps, a final anti-enrofloxacin dilution (1:440) was selected according to the obtained responses, considering sufficiently high response without baseline build up. Next, enrofloxacin concentration measurements were performed in HBS-EP buffer, full fat milk and porcine serum. Enrofloxacin standard solutions were prepared in HBS-EP buffer at concentrations ranging from 0.005 to10000 ng mL^{-1} and each was mixed with anti-enrofloxacin (1:220). These mixtures were injected over the sensor chip in duplicate, starting with blank solution containing only the antibody. The measurement cycle included sample injection (7 minutes contact time) and one injection of regeneration solution (1 minute contact time). For measurements in complex matrices, full fat goat milk and porcine serum were ten times diluted in HBS-EP buffer to prevent high bulk responses which would shift the SPR angle outside of the instrument's scanning range. 1 g of full fat goat milk powder was dissolved in 9 mL of HBS-EP buffer, stirred for 0.5 hr at RT and diluted ten times in HBS-EP buffer and porcine serum was directly diluted ten times in HBS-EP buffer, no filtering or centrifugation steps were used. Enrofloxacin standard solutions in diluted milk and serum were prepared and measured in the same way as in buffer on the same sensor chip. Relative binding (B/B_0) was calculated by dividing the response of the enrofloxacin containing solution (B) by the response of the blank solution (B_0). To generate calibration curves, B/B_0 values were plotted against enrofloxacin concentrations. The calibration curves were fitted with a non-linear 4-parameters (4P) model using GraphPad Prism software ver. 5.02 (GraphPad Software Inc, San Diego, USA) and half maximal inhibitory concentration (IC_{50}) was interpolated. Additionally, immunoassays were characterized by the limits of detection (LODs) which were calculated by subtracting three standard deviations from the average maximum response of the blank solution and by the dynamic measurement ranges, which were set between 0.2 and 0.8 B/B_0.

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Chapter 8

Conclusions and Future Perspectives

This chapter provides an outlook on the research performed within the scope of this thesis and a perspective on future research on iSPR-based systems and further development of biosensors dedicated to food safety monitoring.

The main goal of this study was to develop an iSPR–based biosensor, for multiplexed and quantitative detection of different health-threatening compounds in food. Within the scope of this thesis, various aspects of iSPR biosensor development were addressed. In **Chapter 4**, the possibilities to apply IBIS iSPR sensor to food analysis were explored. IBIS iSPR sensor was the first commercial iSPR system to enter the market in 2004; yet, in 2005 at the beginning of this work, the technology was still in its early stage and many adjustments were made in order to achieve reliable and automated measurements. Additionally, very little knowledge about imaging iSPR analytical capabilities was available in literature. At that time, only a few papers described custom made iSPR systems, which all differed in optical configurations, fluidic systems and sensor chip surfaces [1,2,3]. Thus, the first task was to evaluate the performance of IBIS iSPR system with respect to concentration measurements of high and low molecular weight compounds. Initially the intrinsic optical properties of the sensor were considered. IBIS iSPR optics demonstrated uniform sensitivity across the microarray, suitable LOD for biomolecular interaction measurements and robustness. However, estimation of immobilized amount, under current optical settings of the instrument, was not possible due to high baseline variability. Since the sensor ship is microarrayed outside of the iSPR machine, the ligand immobilization process can not be monitored in real-time. The immobilized amount on each spot could in principal be calculated by subtracting the iSPR angle before and after the spotting. However, since the round sensor chip cannot be placed in exactly the same orientation after the spotting as before the spotting, such a calculation is not accurate. This heterogeneity of the baseline SPR angles over the imaged surface is caused most probably by uneven surface illumination. The inability to monitor the immobilization process in-real time is an inherent disadvantage of this imaging SPR system unlike other iSPR systems (such as ProteON, Bio Rad) that employ flow for ligand immobilization. For this reason, throughout this thesis, the efficiency of ligands immobilization was estimated qualitatively by eye from the image of the sensor chip surface acquired by the camera after spotting. The ligand immobilization efficiency plays a crucial role in concentration measurements based on SPR biosensors. Usually, a maximum load of the sensor surface is desired, in order to reach high assay sensitivity. In microarray iSPR format, immobilization of a sufficient number of molecules with high molecular weight per spot proved to be difficult, due to the nature of the contact spotting procedure. Low spot load is especially problematic when the detection is based on the direct immunoassay format, and in such cases a different spotting procedure should be

considered. As was demonstrated with a direct assay for bovine IgG detection, when the antibodies were immobilized by flow in Biacore 3000 at a sufficient immobilization level, the assay performed well, as opposed to when the antibodies were microarrayed on the sensor chip with the contact spotter or when the antibodies were immobilized at low levels in Biacore 3000. iSPR microarray biosensor for detection of small molecules, based on a competitive immunoassay format, was sensitive at ng mL^{-1} levels and robust. It displayed higher sensitivity than the previously established assay in Biacore 3000. Overall, the initial findings showed that the IBIS iSPR sensor could be a promising tool for concentration measurements of food relevant analytes. Immobilization of high molecular weight compounds in spotting format needed a solution, and was addressed at a later stage (Chapter 6).

Further research, as described in **Chapter 5**, focused on the implementation of an imaging SPR-based biosensor to quantitative measurements of antibiotic residues in milk. Seven model compounds (neomycin, gentamicin, kanamycin, streptomycin, enrofloxacin, chloramphenicol and sulfamethazine) were simultaneously detected via multiplexed competitive immunoassay, in buffer and in milk, using one sensor chip. The immobilization of small molecules was previously proved to be possible with the contact spotter, as described in Chapter 4. All the chosen target compounds for immobilization contained primary amine groups allowing uniform immobilization chemistry on the hydrogel sensor chip via commonly used EDC/NHS chemistry. Still, the immobilization conditions for each individual compound had to be carefully optimized in order to combine the necessary conditions for compound solubility, efficient immobilization and spot formation on the hydrogel surface. The initial selection of the target antibiotic compounds was narrowed down to those described here due to spotting limitations. Thus, even though contact printing (etc. Microgrid) is an established method for DNA, protein and low molecular weight compounds microarraying, for the SPR-based sensors it is rather restrictive. Alternative arraying methods implementing an individual fluidic spot approach, such as Continuous Flow Microspotter (Wasatch microfluidics), have an advantage in such cases (see Chapter 6). Neomycin, kanamycin, streptomycin, enrofloxacin and sulfamethazine detection was achieved via multiplexed competitive assays and for some compounds proved to be sensitive enough for milk control at MRL levels. The cross-reactivity of anti-kanamycin with kanamycin B and tobramycin, anti-sulfamethazine with another 17 sulfonamides and cross-reactivity of anti-enrofloxacin with another four fluoroquinolones broadened the potential screening range of the sensor

chip to 30 antibiotic residues. A variable effect of the milk matrix on the measurements was observed, suggesting that the interference was immunoassay dependent. The overall performance of the microarray biosensor based on iSPR was comparable to that reported for conventional Biacore-based biosensors with four flow channels. Rapid, simultaneous and label free detection of model compounds from four different kinds of antibiotics: aminoglycosides, fluoroquinolones, fenicols and sulfonamides, without complex sample preparation, was achieved. The effects of different food matrixes on the biosensor performance should be further studied by measuring these antibiotic residues in differently processed milk products (e.g. whole milk, ultra-heat treated (UHT) and non UHT, bovine ver. goat). Additionally, for successful implementation of this biosensor for milk monitoring, the screening range has to be extended and ,in particular, antibiotics from the β-lactam family have to be included, since they are the most common veterinary drugs used for mastitis treatment in lactating cows [4]. Implementation of a CFM spotter can broaden the screening range further by enabling application of a different immobilization chemistry on each spot. Simultaneous detection of drug residues with fundamentally different properties, will be a significant step forward in analytical techniques.

Following successful application of the iSPR biosensor to low molecular weight compound detection, the iSPR biosensor was challenged with a direct detection of high molecular weight compounds. **Chapter 6** describes a direct on-chip food allergen screening using iSPR applied to food profiling. To overcome immobilization limitations of contact spotters, in this study a CFM spotter was used. The CFM spotter applies a microfluidic interface to enable antibody immobilisation on each spot individually, offering many advantages over the conventional spotting techniques, including high-quality spot formation on hydrophilic surfaces and a substantial increase in the spot load [5]. Even if only a fraction of the immobilized antibodies is active towards the analyte, the response will be sufficient, enabling direct spotting of polyclonal antibodies without prior affinity purification. This also proved to be extremely useful during the antibodies screening process. Multiple allergen detection was achieved using a direct multiplexed immunoassay. Seven major allergens - peanut, milk, lupine, soy, egg, hazelnut and almond, and six additional tree nut allergens- cashew nut, brazil nut, pine nut, pecan, macadamia nut and pistachio nut were simultaneously detected with analytically relevant sensitivity (low-ppm range), comparably to commercially available ELISA kits. The applicability of the iSPR-based allergen screening was validated by analysing commercially available food samples, cookies and dark chocolates, which were previously

used in an EU survey. Food profiles obtained with the iSPR biosensor provided detailed allergenic composition of the food product and in some cases suggested incorrect allergenic content declaration on the product label. The analytical capabilities of iSPR, demonstrated here, place this technology in a strong competitive position as a powerful analytical technique. The direct on-chip immunoassay format on the iSPR platform, is especially valuable because it enables quantitative detection in a single reagent format. Besides the microarrayed sensor chip, there is no need for any additional bioreagents. This type of application has the lowest costs and the highest potential for in-line and field applications. Different sensor chips dedicated to analysis of different food products (for instance plant protein adulterants in milk products or toxins in cereals) can be developed in the future using the same approach.

Nonspecific binding (NSB) to the sensor chip surface is considered to be one of the main disadvantages in application of systems based on SPR to complex samples analysis [6]; hence an attempt to solve this problem was included in this study. **Chapter 7** describes a novel approach to tackle this drawback by engineering the sensor chip surface for simultaneous sample pretreatment and analysis. A PEO filtering layer with submicron pores, was constructed on top of carboxymethylated dextran (CM-dextran) in order to reduce nonspecific binding to the sensor chip surface in the SPW probed field by filtering out large particles in the sample matrix. This engineered sensor chip surface, named PEO-pores, was evaluated by direct comparison to the regular CM-dextran surface with respect to ligand immobilization, effect on the ligand-analyte interaction and effect on the performance of an analytical assay for antibiotic detection in complex matrices. Even though PEO covered 95 % of the sensor chip it did not compromise neither immobilization nor molecular interaction of high and low molecular weight compounds, due to the availability of the other PEO-uncovered 5 percent of CM-dextran. The performance of the assay for antibiotic detection was significantly improved when performed on the PEO-pores, demonstrating reduced nonspecific binding, higher robustness and higher sensitivity. PEO-pores showed a promising ability to reduce negative matrix effects, improving the assay's sensitivity which could make the difference between a theoretically applicable and actually working assay. The ability to improve the assay's sensitivity in complex matrices by simultaneous on-chip sample preparation also compensates for the reduction in sensitivity when compared to immunoassays where signal amplification is used (e.g. ELISA). This approach of sample pre-treatment on chip, is beneficial not only to SPR-based assays, but also to other optical evanescent wave-

based sensor devices suffering from the similar drawback. Since the source of NSB is matrix dependent, and often is unidentified, future work should focus on challenging the PEO-pores with additional complex matrices, attempting to measure in previously failed samples (such as metabolites in microsomal extracts). Additionally, the sensor chip surface can be similarly engineered to match a specific application by varying PEO-pore sizes.

To summarize, iSPR-based multi-analyte on-chip screening presents a cornerstone in food analysis. It allows multi-analyte and high-throughput monitoring of food production equipment and food products and its routine application will contribute to correct product labelling, adequate legislation and -, foremost, safeguarding the health of consumers. With this approach, each food sample can be analysed within several minutes, faster than any other method currently available, providing a detailed and quantitative food profile. Such a device is highly relevant for multi-analyte screening of various food contaminants and combines the advantages of both an SPR biosensor and a high throughput analytical system. Efficient immobilization of ligands in a microarray format on the sensor chip, and sensitivity and specificity of the antibodies play crucial roles in the utilization of a multiplex immunoassay on the iSPR platform. Thus, both the availability of good bioreagents and adequate spotting techniques will determine the eventual application range of this biosensor. Additionally, the ability to control the NSB by means of sensor chip surface engineering or simple sample preparation will determine the type of food products which can be properly analysed with this technique. Even though high multiplexing capabilities and multiple measurements using a single chip contribute to reduction in the analysis costs, in order to achieve a wider dissemination of the technology, the costs of the iSPR instrumentation need be reduced.

Generally speaking, biosensors offer several major advantages over traditional methods for food analysis. Since food hazards include many fundamentally different agents, e.g. microorganisms, proteins and small molecules, the conventional techniques used for their detection require specialized laboratories and personnel. For example, pathogens are detected by culturing and biochemical techniques, whereas antibiotic residues are detected using high pressure liquid chromatography (HPLC) and mass spectrometry (MS) techniques [7-8]. Biosensors offer a versatile and generic platform, based on bioassays, for detection of a wide variety of the hazardous agents. With one biosensor system (e.g. iSPR) food products can be screened for bacterial pathogens, allergens and antibiotic residues. Biosensors also reduce significantly the costs of bioreagents, due to

miniaturization of the assay, increase robustness and reduce labor due to automation. Portable biosensors, offer a possibility to perform infield measurements [9,10]. However in this respect, iSPR-based biosensors still lag behind the other sensors. In the near future, hand-held, portable, inexpensive, sensitive, and easy-to use biosensing devices will be developed [11]. Stepping outside the analytical laboratory, the biosensors will dominate the infield food and environmental monitoring, providing cost-efficient and rapid analytical solutions and thus contributing to continuous improvement of life quality in both industrialized and developing countries.

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Summary

Food safety is an increasing health concern, recognised and promoted by many institutions across the globe. Food products can be contaminated with pathogenic microorganisms, environmental pollutants, veterinary drug residues, allergens and toxins. Public health concerns which have been raised in relation to hazardous agents found in food include, among others, increased cancer risk, endocrine, reproductive and neurobehavioral systems disruption, teratogenesis, antibiotic resistance and even death in cases of allergic reactions and acute poisoning. Some of the food hazardous agents (e.g. pathogenic microorganisms and toxins) can even be used as biological warfare, spread through food and agricultural chains. Thus, an adequate detection of these compounds is also important for biosecurity. In order to safeguard consumers' health, legislations have been put in place both in the US and the EU. These laws specify for each health threatening compound the maximal acceptable amounts in different food products. Besides health issues, food safety and quality has an economical impact on the food industry, where quality control expenses amount to about 1.5 – 2 % of the total sales. Since more and more food products nowadays contain multiple and processed ingredients, which are often shipped from different parts of the world, and share common production lines and storage spaces, food safety and quality monitoring becomes a challenging task. Traditional analytical methods require dedicated laboratories, equipment and highly trained personnel for detection and identification of each type of hazardous agent (e.g. antibiotics, bacteria, allergens). These techniques are also time-consuming and often expensive. There is a growing need for multi-analyte screening methods, which will enable rapid and simultaneous detection of multiple compounds in complex food samples. In recent years, biosensors have been applied successfully to food analysis, incorporating the same bioassay principals as traditional methods with transducers (optical, electrochemical, etc) in novel, usually miniaturized, integrated analytical devices. However, most of these biosensors still lack the desired level of the multiplexicity.

Recent developments in the field of Surface Plasmon Resonance (SPR) technology in the direction of high-throughput systems and multi-analyte measurements present a promising alternative for the existing systems. One of such systems is imaging SPR (iSPR); it enables real-time and label free read-out of spatially modified surfaces (e.g. microarrays). The aim of this study was to develop an iSPR–based biosensor, for

simultaneous and quantitative detection of different health-threatening compounds in food. To obtain a comprehensive overview on the analytical applicability of such a system, several points were addressed. The intrinsic sensor properties, such as optical sensitivity and robustness, of the iSPR instrument were studied. Further on, both direct and competitive immunoassay formats for high and low molecular weight compounds detection using the iSPR platform were evaluated. Then, the iSPR-based biosensor was applied for detection of regulated substances in food such as antibiotic residues in milk and allergens in cookies and chocolates. Finally, the most common drawback of using SPR for screening in complex biological matrices, the nonspecific binding to the sensor chip surface, was tackled. The sensitivity of both high and low molecular weight compounds was proven to be sufficient for some of the hazardous agents detection at the maximum residue levels, established in the EU legislation, as was demonstrated by simultaneous detection of seven antibiotic residues in milk and twelve allergens in cookies and dark chocolates. The analysis time takes about 10 minutes and provides quantitative information on multiple targets, producing a fingerprint (allergenic fingerprint for instance) of the tested food. This detailed food profile contributes to the decision making process on the quality and safety of foods, basing it on the total picture of all target compounds present. In order for iSPR-based biosensing to reach its full potential and to become a widely applied routine analytical tool, the instrumental cost needs to be reduced and the analysis further simplified, becoming cost-effective and approachable to non-trained personnel. An additional drawback in analytical applications of a SPR sensor is the nonspecific binding of the matrix components of complex samples to the sensor surface. Many assays based on SPR fail due to inapplicability to measure in “real” samples. As a possible solution to this problem, sensor chip surface engineering was suggested in this thesis. A nanopatterned filter layer covering the sensor chip surface was found to be effective in reducing nonspecific binding when the measurements were performed in “raw” samples by keeping the non-soluble aggregates and big sample matrix components beyond the sensing region of the SPR. With respect to other existing biosensors, iSPR still lags behind in terms of sensitivity and portability.

In summary, the results of this study demonstrate that iSPR-based biosensor is a versatile platform, which can be applied for a wide variety of fundamentally different analytes and offers several advantages over already existing methods. SPR detection principle eliminates the need in labelling and the instrumental set-up allows automated analysis. High multiplexing capabilities and short measurement times are obtained with no

need for complex and time consuming sample preparation steps. By using iSPR-based biosensor, one can obtain robust and quantitative information on the target analyte concentration, in real time and with high specificity (or broad spectrum, depending on the assay). In conclusion, on-chip screening using iSPR, described here, presents a powerful analytical approach towards food safety and quality monitoring which satisfies the current need in rapid and multi-analytical devices.

Samenvatting

Voedselveiligheid wordt wereldwijd erkend als belangrijk voor de gezondheid. Voedingsmiddelen kunnen besmet zijn met ziekteverwekkende micro-organismen, milieuverontreinigingen, residuen van (dier)geneesmiddelen, allergenen en toxines. Gevaren voor de volksgezondheid die worden veroorzaakt door stoffen in levensmiddelen zijn onder andere: grotere kans op kanker, verminderde vruchtbaarheid, aantastingen van de endocriene klieren en van het centrale zenuwstelsel, foetale beschadigingen (teratogeniciteit), verhoogde weerstand tegen antibiotica, en allergische reacties en acute vergiftiging, eventueel met dodelijke afloop. Sommige gevaarlijke stoffen (pathogene micro-organismen en toxines) kunnen zelfs via de agri-food keten worden gebruikt voor bioterrorisme en biologische oorlogsvoering en een toereikende detectie van deze stoffen is belangrijk voor de veiligheid. Met het oog op genoemde zaken is zowel in Europa als in de VS wetgeving betreffende de gezondheid van consumenten ontwikkeld. Voor elk van de gezondheidsbedreigende stoffen is een wettelijk toegestane maximale hoeveelheid in verschillende voedingsmiddelen gespecificeerd. Handhaving van voedselkwaliteit en –veiligheid is ook van groot economisch belang: de kosten van de kwaliteitscontrole bedragen ongeveer 1.5 – 2 % van de totale omzet. Omdat tegenwoordig steeds meer voedingsmiddelen zijn samengesteld uit meerdere en bewerkte ingrediënten, die vaak uit verschillende delen van de wereld afkomstig zijn en gemeenschappelijke productielijnen en opslagruimtes gedeeld hebben, is bewaking van voedselkwaliteit en –veiligheid een uitdagende bezigheid.

Voor de traditionele analyse van stoffen als antibiotica, allergenen, etcetera, zijn goed uitgeruste apparatuur en laboratoria vereist, alsmede hoog opgeleid personeel. De gebruikte methoden zijn vaak tijdrovend en duur. Er is een toenemende behoefte aan methoden waarmee snel meerdere stoffen tegelijkertijd kunnen worden aangetoond en geïdentificeerd (zgn. “multi-analyte” opsporingsmethoden) in complexe voedingsmiddelen. De laatste jaren is in de levensmiddelenanalyse succesvol gebruik gemaakt van geminiaturiseerde biosensoren, waarbij de biochemische reacties worden uitgelezen d.m.v. een optisch of electrochemisch signaal. De meeste van dergelijke biosensoren schieten echter nog tekort wat betreft het aantal tegelijkertijd aan te tonen stoffen.

Recente ontwikkelingen van de Surface Plasmon Resonance (SPR) technologie m.b.t. snelle multi-analyte bepalingen bieden een veelbelovend alternatief voor bestaande methoden. Imaging SPR (iSPR) is zo’n nieuwe ontwikkeling; met behulp van micro-arrays van verschillende reagentia op het oppervlak van een sensorchip kunnen meerdere stoffen tegelijkertijd worden aangetoond zonder gebruik te maken labels.

Het doel van het in dit proefschrift beschreven onderzoek was een op iSPR gebaseerde biosensor te ontwikkelen voor gelijktijdige kwantitatieve bepaling van verschillende gezondheidsbedreigende stoffen in voedingsmiddelen. Om goed inzicht te krijgen in de toepassingsmogelijkheden van zo'n systeem zijn verschillende aspecten bestudeerd, zoals optische gevoeligheid en robuustheid. Ook is nagegaan of iSPR geschikt is om zowel directe als competitieve immunoassays uit te voeren voor stoffen met een hoog en laag molecuulgewicht. Vervolgens zijn (wettelijk gereguleerde) stoffen in voedingsmiddelen bepaald, t.w. residuen van antibiotica in melk en allergenen in koekjes en in chocolade. Ten slotte is de niet-specifieke binding, één van de meest voorkomende nadelen van de toepassing van SPR in complexe biologische materialen, aangepakt. Zeven verschillende antibioticaresiduen in melk en twaalf allergenen in koekjes en chocolade konden tegelijkertijd worden aangetoond met een gevoeligheid die groot genoeg is om aan de in de EU wetgeving vastgestelde maximaal toegestane hoeveelheden te voldoen. In ongeveer 10 minuten kon zo een kwantitatief beeld van de aanwezigheid van allergenen en antibiotica in de monsters verkregen worden. Zulke gedetailleerde informatie vergemakkelijkt besluitvorming m.b.t. de kwaliteit en veiligheid van voedsel. Echter, voordat iSPR biosensoren als routine-instrument gebruikt zullen worden, moet de apparatuur goedkoper worden en de analysemethode verder vereenvoudigd en gebruikersvriendelijker worden. Een bijkomend nadeel is de niet-specifieke binding van stoffen uit complexe monsters aan het sensoroppervlak. In dit proefschrift wordt beschreven hoe bedekking van het oppervlak door een filter met nanoporiën niet-specifieke binding van moleculaire aggregaten en grote moleculen kan onderdrukken. Toch, vergeleken met andere biosensoren, is iSPR nog steeds de mindere wat betreft gevoeligheid en draagbaarheid.

Samenvattend kan gezegd worden dat de resultaten van het in dit proefschrift beschreven onderzoek laten zien dat een op iSPR gebaseerde biosensor een veelzijdig platform biedt voor het in korte tijd testen op de aanwezigheid van een groot aantal uiteenlopende stoffen en dat het veel voordelen heeft boven reeds bestaande analysemethoden. Detectie d.m.v. SPR kan gemakkelijk geautomatiseerd worden en er zijn geen labels nodig. Bovendien zijn ingewikkelde en tijdrovende monstervoorbereidingen niet nodig. Met iSPR kunnen concentraties van de aan te tonen stoffen kwantitatief en met een hoge specificiteit vastgesteld worden, of kan, afhankelijk van de gekozen assay, een breed spectrum aan stoffen doorgemeten worden. Kortom, de op iSPR gebaseerde multi-analyte biosensor, zoals beschreven in dit proefschrift, is een krachtig hulpmiddel bij het bewaken van de voedselkwaliteit en –veiligheid en voldoet daarmee aan de hedendaagse behoefte aan snelle "multi-analyte" methoden.

סיכום

כיום נושא בטיחות המזון זוכה לתשומת לב רבה מצד ארגוני הבריאות המתייחסים אליו כאל בעיה בריאותית כלל עולמית. מזון עלול להכיל מספר גורמים המסכנים את בריאות הצרכן, הנפוצים בינהם כוללים אורגניזמים גורמי מחלות, מזהמים סביבתיים, שאריות תרופות וטרינריות, אלרגנים ורעלנים. סכנות בריאותיות המיוחסות לגורמי סיכון במזון כוללים סוגי סרטנים שונים, הפרעות במערכת ההורמונלית, הרבייה והעצבית, טראטוגנזה, עמידות לאנטיביוטיקה ואפילו מוות במקרים של שוק אנפילקטי והרעלות אקוטיות. על מנת להגן על בריאות הציבור ברוב המדינות המפותחות חוקקו חוקים המפרטים עבור כל גורם סיכון את הרמות המרביות המותרות במזונות השונים. בנוסף, לנושא בטיחות ואיכות המזון יש השפעה כלכלית על תעשיית המזון, אשר מוציאה עד כ-2% מכלל הכנסותיה על תהליכי בקרת איכות.

בקרת איכות ובטיחות המזון הפכה למשימה מאתגרת, מאחר והמזון המיוצר כיום מכיל מרכיבים מעובדים, אשר מיובאים מכל רחבי העולם וחולקים את פסי היצור ואזורי האחסון. שיטות אנליטיות מסורתיות ,הנמצאות בשימוש כרגע, דורשות מעבדות, ציוד וכוח אדם מתמחה על מנת לזהות כל סוג של גורם סיכון בנפרד. יתר על כך, רוב השיטות הללו הינן יקרות ודורשות זמן רב ועבודה אנטנסיבית לקבלת תוצאות האנליזה. לכן, כיום יש צורך הולך וגובר בשיטות מולטי-אנליטיות לסריקה מהירה וסימולטנית של סוגים שונים של גורמי סיכון במזון. אחת החלופות לשיטות המסורתיות היא שימוש בביו-חיישנים ,אשר מיישמים את אותם העקרונות כמו בשיטות מסורתיות, בשילוב עם משדרים (אופטיים, אלקטרוכימיים וכו') המורכבים יחד כרכיבים אנליטים חדשניים, בדרך כלל ממוזערים. חסרון רוב הביו-חיישנים הקיימים הוא שלא ניתן לבדוק מספר רב של גורמי סיכון במקביל ולכן הם טרם מספקים פתרון ברמת המולטי-אנליזה הנדרשת.

מטרת המחקר המתואר בתזה זו היתה לפתח שבב לזיהוי כמותי וסימולטני של גורמי סיכון שונים במזון. השיטה שפותחה מבוססת על ביו-חיישן מסוג (iSPR) imaging Surface Plasmon Resonance. טכנולוגיית ה-iSPR מאפשרת קריאת סיגנל קישור בין מולקולות ממשטחים מעובדים מרחבית (כגון מיקרו-מערכים) בזמן אמת וללא צורך בסימון. במסגרת המחקר אופיינו היכולות האנליטיות של המערכת ברמת הרגישות האופטית וחוסן המערכת כמו כן הוערכה המערכת כמתאימה לשימוש באנליזה בדוגמאות מזון של גורמי סיכון ,הן בעלי משקל מולקולרי גבוה והן בעלי משקל מולקולרי נמוך. כשלב שני פותחו שני שבבים לזיהוי שאריות אנטיביוטיקה בחלב ואלרגנים בעוגיות ושוקולד. על מנת לזהות אניטיביוטיקה בחלב נעשה שימוש בנוגדנים המזהים מרכיב אנטיביוטי יחיד או קבוצה של מרכיבים. האנטיביוטיקות הודפסו על השבב ובעזרת כימות סיגנל הקישור לנוגדן בתמיסה בנוכחות אנטיביוטיקה בדוגמא הצלחנו למדוד סימולטנית שבע אנטיביוטיקות המשתייכות לארבע קבוצות שונות,זאת תוך מספר דקות ועם רגישות של חלקיקים לביליון. עבור שבב האלרגנים, השתמשנו במדידת קישור ישיר בין האלרגן בתמיסה לבין הנוגדן שמודפס על פני משטח השבב. באופן זה הצלחנו למדוד שנים-

עשר אלרגנים עיקריים במוצרי עוגיות ושוקולדים כהים מיצרנים שונים המכילים מעל 70% מוצקי קקאו .נדרשו שבע דקות לזיהוי האלרגנים על ידי השבבברגישות של מספר חלקיקים למליון. יתרון חשוב במדידת קישור ישיר בשבב האלרגנים הוא שבפורמט זה אין צורך בשום אלמנטים נוספים מלבד השבב והדוגמא, דבר שמאפשר שילוב השבב בפסי ייצור למטרת בקרה בזמן אמת. בנוסף לשני שבבים אלה, הוצע בתזה זו פתרון חדשני לבעיית הקישור הלא ספציפי שנפוץ במערכות דומות. בעת מדידה בדוגמאות מורכבות כמו חלב מלא או סרום, מקרים רבים קישור לא ספציפי של מרכיבי הדוגמא ממסך סיגנל של הגורם הנבדק. מסיבה זו, ביו-חיישנים רבים נכשלים במעבר ממדידות בבופר לדוגמאות אמיתיות. בעבודה זו הונדס ננו-מסנן מעל לשכבת הפולימר שבתוכו מתרחשת האינטרקציה. עקרון הפעולה של ננו-מסנן מתבסס על מניעת מרכיבי דוגמא גדולים כמו אגרגטים או מיצלות מלהגיע לאיזור שבו נמדד סיגנל הקישור על ידי ה-SPR. ננו-מסנן זה נמצא יעיל במניעת קישור לא ספציפי בעת מדידות של ריכוזי אנטיביוטיקה בחלב מלא ובסרום לא מטופל.

לסיכום, במסגרת העבודה המתוארת בתזה זו פותח ביו-חיישן על שבב לניטור שאריות אנטיביוטיקה בחלב ואלרגנים בעוגיות ושוקולד. בנוסף, הונדס משטח החיישן על מנת למזער את הקישור הלא ספציפי שנפוץ במדידות בדוגמאות מזון וסרום לא מעובדות. הממצאים הראו שרגישות המערכת עבור זיהוי תרכובות הן בעלות משקל מולקולרי גבוה והן משקל מולקולרי נמוך מספיקה על מנת לנטר גורמי סיכון במזון ברמות המוגדרות בחוקי האיחוד האירופאי. זמן האנליזה במערכת הנ"ל הינו דקות ספורות ובמהלך המדידה מתקבל פרופיל רב כמותי של המרכיבים המנוטרים אשר מספק טביעת אצבע של המזון הנבדק (למשל הפרופיל האלרגני). הפרופיל המתקבל מדוגמאות המזון מסיע בהליך קבלת ההחלטות בנושא בטיחות המזון ומבסס אותו על תמונה כוללת של כל גורמי הסיכון הנבדקים. כדי למצות את הפוטנציאל הגלום בביו-חיישן מסוג זה ובכדי להפכו לכלי אנליטי המיושם באופן רחב בצורה שגרתית הנגיש לכוח אדם ללא התמחות ספציפית ,יש צורך בקיצוץ דרסטי במחיר המכשיר ובפישוט הליך האנליזה. בהשוואה לסוגי חיישנים אחרים בולטים חסרונות טכנולוגיית ה-iSPR בתחום רגישות וניידות המערכת, למרות זאת, ביו-חיישנים מבוססי iSPR הינם פלטפורמה אנליטית מגוונת ,יעילה לאיתור תרכובות שונות ומציעה מספר יתרונות על המערכות הקיימות כמפורט להלן. עקרון החישה של אירועי קישור על המשטח בעזרת SPR מבטל את הצורך לסימון המולקולות והמכשיר מאפשר אוטומטיזציה של הליך האנליזה. בנוסף, המערכת מתאפיינת ביכולות רבוב גבוהות ,זמני מדידה קצרים ומדידה בדוגמאות מורכבות ללא צורך בשלבי הכנת הדוגמא אשר בדרך כלל מייקרים ומאריכים את תהליך האנליזה. שימוש בביו-חיישן מסוג iSPR מאפשר קבלת מידע כמותי ורובסטי על ריכוז התרכובת הנבדקת, בזמן אמת ובספציפיות גבוהה. שימוש ב בביו-חיישן מסוג iSPR המתואר בתזה זו מדגים יכולת אנליטית מבטיחה לבקרה על בטיחות המזון אשר עונה על הצרכים הקיימים להתקנים מהירים ומולטי-אנליטים.

Curriculum Vitae

Sabina Rebe was born on October the 3rd 1977 in Riga, Latvia . In 1990, together with her family, Sabina moved to the city called Beer-Sheva, in the middle of the Israeli desert. In 1995, she graduated from high school majoring in biology and mathematics. Half a y ear later, she joined the Israeli Army for compulsory service, which she successfully completed after 21 months with the rank of sergeant in the Air Force.

Technion Israel Institute of Technology

Considering software engineering as a main career orientation she signed up for undergraduate studies in Technion – Israeli Institute of Technology in 1998. Eventually, she got her B.Sc. in Food Engineering and Biotechnology, with dean's and presidential excellence acknowledgments for academic achievement, graduating ahead of time in April 2002. She performed her final research project in the group of Prof. A. Reznik at The Bruce Rappoport Institute for Research in the Medical Sciences, Haifa, Israel. She studied the ability of antioxidants, integrated in to the cigarette filter, to minimize the damaging effect of the cigarette smoke on body fluids.

In June 2002 Sabina joined the R&D department in Procognia ltd. (Israel). There she was involved in lectin array-based platform development for high-throughput glycan-profiling of intact proteins. After a while, she resumed her studies towards a Master degree, realizing that school is not over yet. Under a supervision of Prof. B. Solomon, at Biotechnology department in Tel-Aviv University, she worked on the application of deglycosylated catalytical antibodies to Alzheimer's immunotherapy. In 2004 Sabina obtained M.Sc. in Biotechnology with Summa Cum Laude, and rejoined Procognia as a full time researcher.

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As much as she enjoyed her work in Procognia, she felt like its time for a change, so in October 2005 she moved to the Netherlands dragging along her boyfriend and her dog. Settling down in de Nude, Wageningen, she started her PhD in Wageningen University under the supervision of Prof. Willem Norde and Dr. Monique Bremer in Rikilt-Institute of Food Safety. During her PhD she was involved in a "Screen Chip" project in cooperation with Twente University. The aim of this project was develop a microfluidic biochip for multi-analyte food screening. Her main responsibility was to set up iSPR-based multi-analyte immunoassays for food screening, which could later on be integrated with the microfluidic interface developed in Twente.

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This booklet describes the scientific out-come of her PhD studies. Besides conducting a research, she took part in post-graduate courses, scientific conferences and meetings. She also assisted in teaching practical courses in the university and supervised Master students during their thesis research.

It turned out that Wageningen is a good place to raise kids. Her son, Benjamin and daughter Julia were born during her second and third year in the PhD, so the studies were prolonged to 5 years. Currently Sabina occupies a Post Doctoral position in Rikilt-Food Safety Institute, developing SPR-based screening method for detection of engineered nanoparticles in food within NANOLYSE EU project.

List of Publications

- **Sabina Rebe Raz,** Willem Haasnoot," Multiplexed Bioassay-based Approaches to Food and Environmental Contaminants Analysis", submitted to Advances in Chemical Research.
- **Sabina Rebe Raz,** Gerardo R. Marchesini , Maria G.E.G. Bremer , Pascal Colpo, Guido Giudetti, Willem Norde, Francois Rossi, "Nanopatterned Submicron Pores on a Gel-supported Membrane for On-chip Sample Preparation in Surface Plasmon Resonance Sensing", Submitted to Lab on a Chip.
- **Sabina Rebe Raz,** Hong Liu, Willem Norde, Maria G.E.G. Bremer, "Food Allergens Profiling With Imaging Surface Plasmon Resonance-Based Biosensor", accepted for publication in **Analytical Chemistry**.
- **Sabina Rebe Raz,** Maria G. E. G. Bremer, Willem Haasnoot and Willem Norde, " Label-Free and Multiplex Detection of Antibiotic Residues in Milk Using Imaging Surface Plasmon Resonance-Based Immunosensor", **Analytical Chemistry,** 2009, *81* (18), pp 7743–7749.
- **Sabina Rebe Raz**, Maria G.E.G. Bremer, Marcel Giesbers and Willem Norde, "Development of a biosensor microarray towards food screening, using imaging surface plasmon resonance", **Biosensors and Bioelectronics**, Volume 24, Issue 4, 1 December 2008, Pages 552-557.
- Iva Navratilova, Giuseppe A. Papalia, Rebecca L. Rich, Daniel Bedinger, Susan Brophy, Brad Condon, Ta Deng, Anne W. Emerick, Hann-Wen Guan, Tanya Hayden, Thomas Heutmekers, Terry Nakagawa, Fabio Parmeggiani, Xiaochun Qin, **Sabina Rebe**, Nenad Tomasevic, Tiffany Tsang, M. Brett Waddell, Fred Feiyu Zhang, Stephanie Leavitt and David G. Myszka. , "Thermodynamic benchmark study using Biacore technology ",**Analytical Biochemistry**, Volume 364, Issue 1, 1 May 2007, Pages 67-77.
- **Sabina Rebe**, Beka Solomon, "Deglycosylation of anti-ß amyloid antibodies inhibits microglia activation in BV-2 cellular model ", **American Journal of Alzheimer's Disease and Other Dementias®**, Vol. 20, No. 5, 303-313 (2005).

- **(WO/2005/059563)** METHOD FOR ANALYZING A GLYCOMOLECULE ; Inventors: AMOR, Yehudit; (IL). MARKMAN, Ofer; (IL). GULKO, Mirit, Kolog; (IL). AMOKOVLISTKY, Albena; (IL). KLEINMAN, Fredi; (IL). ALERGAND, Tal; (IL); ROSENFELD, Rakefet; (IL); MAYA, Ruth; (IL), **REBE, Sabina**; (IL). KASUTO, Idil, Kelson; (IL). BANGIO, Haim; (IL).
- **(WO/2005/120571)** METHOD OF PASSIVE IMMUNIZATION AGAINST DISEASE OR DISORDER CHARACTERIZED BY AMYLOID AGGREGATION WITH DIMINISHED RISK OF NEUROINFLAMMATION ; Inventors: **REBE, Sabina**; (IL). SOLOMON, Beka; (IL).

Overview of Completed Training Activities, Vlag Graduate School

Discipline-specific activities

- Biosensor Tools-Advanced biosensor tools workshop (2006), Salt Lake city, USA.
- Advances in Microarray Technology (2006), Amsterdam, The Netherlands.
- 9th European Training Course on Carbohydrates (2006), Wageningen, The Netherlands.
- The 8th Workshop on Biosensors and Bioanalytical µ–Techniques in Environmental and Clinical Analysis (2007), Goa, India.
- Fundamentals of Nanotechnology (2007), Enschede, The Netherlands.
- Bio-Polymers (2007), Wageningen, The Netherlands.
- Metabolomics (2007), Wageningen, The Netherlands.
- The First International Workshop on Label-free Biosensing (LFB 2008), Enschede, The Netherlands.
- The 9th Workshop on Biosensors and Bioanalytical µ–Techniques in Environmental and Clinical Analysis (2009), Montreal, Canada.
- Xth International Conference on AgriFood Antibodies (ICAFA 2009), Wageningen, The Netherlands.
- Screening Europe (2010), Barcelona, Spain.
- Biosensors 2010-20th Anniversary World Congress on Biosensors, Glasgow, UK.

General courses

- Time and Project Management (2006), Wageningen, The Netherlands.
- Techniques for Writing and Presenting a Scientific Paper (2006), Wageningen, The Netherlands.
- Orientation on Technopreneurship Entrepreneurial Management (2008), Leiden, The Netherlands.
- Career Perspectives (2009), Wageningen, The Netherlands.
- Teaching and Supervising Thesis Students (2009), Wageningen, The Netherlands.

Optional activities

- STW project-progress half yearly meetings, Enschede /Wageningen, The Netherlands.
- Group meetings-RIKILT, Wageningen, The Netherlands.

Teaching activities

- Chemistry for Life Sciences II -practicals (2006, 2007), Wageningen, The Netherlands.
- Physical Chemistry-practicals (2006, 2007), Wageningen, The Netherlands.

Abstract

This thesis describes on-chip detection of health-threatening compounds in food, using imaging Surface Plasmon Resonance (iSPR) biosensor. iSPR biosensor combines spatial modification of the sensor chip surface with SPR-based detection, enabling label free and multiplexed analysis in a single measurement. Within the scope of this thesis the iSPR biosensor was evaluated for sensitivity, robustness and potential applicability to measurements in food samples. Simultaneous detection of seven antibiotic residues was achieved by combining an antibiotics-microarrayed chip with seven immunoassays in a competitive format. The developed on-chip antibiotic residues detection method showed ppb sensitivities for the multiplexed measurements in milk. Further on, an allergen chip was developed for food allergen detection in cookies and dark chocolates. Here, the chip was microarrayed with specific anti-allergen antibodies and the allergen proteins were detected in the food sample directly. With this chip, 12 major allergens were simultaneously detected with ppm sensitivity in several cookies and dark chocolate samples from different manufacturers. Both methods developed here displayed sufficient sensitivity for food monitoring and short measurements times (below 10 minutes per sample). This thesis also describes a novel approach to tackle the most common drawback of using SPR biosensors for measurements in complex biological matrices - the nonspecific binding to the sensor chip surface. By using a perforated membrane supported by a polymeric gel structure that exceeds the evanescent wave penetration depth, a nano-filter was created. This nano-filter prevented the diffusion of large particles and/or aggregates that bound non-specifically and interfered with the specific analyte detection. On the whole, it was demonstrated that by using iSPR-based biosensors, one can obtain robust and quantitative information on multiple analytes concentration, benefiting from high multiplexing capabilities and short measurement times together with no need for complex and time consuming sample preparation steps.

The research described in this thesis was carried out in the Biomolecular Detection group at RIKILT- Institute of Food Safety, Wageningen, The Netherlands.

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